THE *IN VIVO* AND *IN VITRO* EFFECT OF A FRUCTOOLIGOSACCHARIDE PREBIOTIC COMBINED WITH ALFALFA MOLT DIETS ON EGG PRODUCTION AND *SALMONELLA* IN LAYING HENS

A Thesis

by

LISA MICHELLE DONALSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Poultry Science

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Approved as to style and content by:

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Steven C. Ricke (Chair of Committee)	Leon Kubena (Member)
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ABSTRACT

The *in vivo* and *in vitro* Effect of a Fructooligosaccharide Prebiotic Combined with Alfalfa Molt Diets on Egg Production and *Salmonella* in Laying Hens. (May 2005)

Lisa Michelle Donalson, B.S., Sam Houston State University

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Salmonellosis affects an estimated 1.4 million people a year with a great majority of cases never being reported. *Salmonella* Enteritidis (SE) can be found in a variety of foods including poultry meat and eggs. Susceptibility of SE colonization is increased by molting.

Induced molting is used in the poultry industry to rejuvenate the hen's reproductive tract and increase post molt egg quality and production. The most common molting method is complete feed withdrawal. Recent animal welfare pressures have encouraged the industry to seek alternatives to feed withdrawal with one alternative being feeding a high fiber diet like alfalfa. Alfalfa is high in protein, but low in energy which is desirable for a molt diet. Alfalfa's fermentation properties have been thought to be an inhibitor in pathogen colonization during molting. Including a prebiotic such as fructooligosaccharide (FOS) in the molt diet is thought to further decrease colonization while benefiting the indigenous microflora.

Laying hens were molted using alfalfa combined with different ratios of layer ration in an *in vivo* experiment. The hens responded comparably to the alfalfa molt diets

as they did to feed withdrawal as far as post-molt production parameters were concerned, thus showing that alfalfa was a viable alternative molt diet.

Two *in vitro* studies were designed to evaluate the fermentation properties of alfalfa and layer ration combined with the prebiotic FOS and their abilities to inhibit *Salmonella* growth. Each treatment was combined with diluted cecal contents and allowed to ferment. The results showed that the most fermentation occurred when alfalfa was the substrate and was slightly increased with the addition of FOS. In addition, combining FOS with alfalfa inhibited *Salmonella* growth.

To integrate these results, an *in vivo* study was preformed using an alfalfa/layer ration diet from the previous *in vivo* study with FOS. Volatile fatty acids and lactic acid measurements were made to evaluate fermentation while *Salmonella* colonization was measured in pertinent organs and in fecal shedding. The results of this study further substantiate alfalfa as a molt diet and conclude that the addition of FOS does, while not statistically significant, further inhibit *Salmonella* colonization.

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TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

Salmonellosis is a foodborne disease that affects over 1.4 million people each year in the United States alone, of which more than 500 are fatal (CDC, 2004). Frenzen *et al.*(1999) estimate the annual cost of foodborne salmonella infection is nearly 2.3 billion dollars in the United States. The majority of this cost is due to loss of productivity in the workforce and medical bills (Frenzen et al., 1999). While human *Salmonella* cases are at their lowest levels since 1987, it is not on the decline (Cogan and Humphrey, 2003). The CDC estimates that for every one case that is reported, 37 go unrecognized (2004) thus the total number of outbreaks is much greater and the cost estimates are quite conservative. While there are estimated to be nearly 2,400 different serovars of *Salmonella* believed to cause foodborne illness, two are considered to be the most dominant.

The two serotypes that cause the majority of the cases are *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST). SE cases are generally believed to be derived from shell eggs from chickens. These eggs come from hens that appear perfectly healthy but carry the disease in their gastrointestinal and reproductive tracts, which is then transmitted to the interior of the egg prior to shell formation; in addition, these contaminated eggs are indistinguishable from non-contaminated, normal eggs (Cogan

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This thesis follows the format of Poultry Science.

and Humphrey, 2003; Wegener et al., 2003). This fact along with undercooking contaminated eggs leads to SE infection. Patrick et al. (2004) estimates that of all the outbreaks of SE from 1985 through 1999, 80% were egg associated. Among this 80%, 28% of the outbreaks were from foods that contained raw eggs such as ice cream, egg nog and Caesar salad dressing. Of the outbreaks, 27%were attributed to traditional egg dishes such as omelets, French toast and other foods that use egg batter (Patrick et al., 2004).

While the incidence of SE in egg contents is estimated to be 0.005%, it is still a prominent food safety issue, as approximately 3.2 million eggs are contaminated annually in the United States alone (CDC, 2004). Physiological stresses, such as molting, increase the susceptibility of SE infection in the hen, (Poppe, 1999).

With ever increasing food safety concerns, it is essential to develop methods to alleviate *Salmonella* infection. By understanding the physiology of the hen and the route of transmission of *Salmonella*, it will be easier to implement alternative molt diets. This knowledge will also facilitate incorporating prebiotics into poultry diets, in particular laying hen diets, to benefit the hen while inhibiting *Salmonella* colonization.

CHAPTER II

BIBLIOGRAPHIC REVIEW

MOLTING

Molting in avian species is defined as periodic shedding and replacement of feathers. During this time, most birds also undergo reproductive rejuvenation in which egg production ceases and the reproductive tract regresses (Berry, 2003). Domestic laying hens will naturally undergo a molt after an extensive egg laying period; however this process generally takes approximately four months (North and Bell, 1990), which raises economic concerns as the hens continue to be fed during non productive times (McDaniel and Aske, 2000) .

The molting process can be sped up by a management practice commonly used called an induced or forced molt (other terms include pause, forced rest, and recycling; Berry, 2003). The induced molt method, which was developed in the 1960's, uniformly rests all hens and returns them to a more consistent high rate of lay for an extended period (McDaniel and Aske, 2000). By the mid-1970's induced molting had gained popularity throughout the United States and in many countries around the world (Bell, 2003). The U.S. commercial egg industry commonly uses induced molt procedures to rejuvenate flocks for a second or third laying cycle and to increase profits. According to

Bell (2003), approximately 75% of commercial laying facilities in the United States used an induced molt program in order to rejuvenate flocks for increased productivity.

Implementing an induced molt program can result in a 30% higher profit margin for producers when compared to an all-pullet operation (Bell, 2003). Induced molt management practices increase profits by optimizing the use of replacement pullets, considering a non-molted program would require 47% more hens to keep houses at maximum capacity (Bell, 2003). In addition to increased profit margins, an induced molt rejuvenates the hens' reproductive tract to produce higher quality eggs which are more marketable (Keshavarz and Quimby, 2002). The main purpose of molting is to cease egg production in order for the hens to enter a non-reproductive state which increases egg production and egg quality post molt (Webster, 2003). On the average, laying hens undergo an induced molt at 65-70 weeks of age and commonly return to egg production, mortality, and egg quality values seen in hens aged 40 to 50 weeks of age (Bell, 2003). An induced molt it seen as advantageous because hens are uniformly molted and can return to 50% production in less than 6 weeks (Parkhurst and Mountney, 1988).

PHYSIOLOGY OF A MOLT

An induced molt is usually initiated by decreasing the photoperiod from 16 hours light: 8 hours dark to 8 hours light : 16 hours dark (Andrews et al., 1987b) a week prior to removing feed which allows for continued production while the birds are

photosensitized in order for a more complete and rapid molt after fasting (Andrews et al., 1987b). Changing day length by either increasing or decreasing it, causes changes in circadian and circannual rhythms. The reduction in photoperiod has been proven to initiate molt and is related to a more complete molt. Reducing photoperiod acts on the hypothalamic-hypophyseal axis (Andrews et al., 1987b) and initiates gonadal regression (Berry, 2003). Upon the loss of gonadotrophin, ovaries regress and the follicles become atretic while the yolk is resorbed. During this time, ovary weight decreases thus decreasing overall body weight. In addition, a week after the photoperiod is reduced, feed is removed or the diet is changed to a low energy molt diet, decreasing adipose deposits and overall body weight (Brake, 1993).

Optimal body weight loss during a molt is between 27 to 32%, and at this time the shell gland lipid decreases (Brake, 1993). Shell gland lipid naturally increases as a hen ages causing an increase in shell-less eggs; however, once 25% body weight loss is achieved, lipid accumulation in the uterus is decreased (Brake, 1993; Berry, 2003), consequently decreasing the incidence of shell-less eggs and increasing postmolt production. In addition, molting increases the concentration of shell gland calcitriol receptor and calbindin which are responsible for increased shell strength post molt (Brake, 1993).

During ovary regression, ovarian steroid synthesis of estradiol and progesterone is decreased thus decreasing the synthesis of yolk precursors in the liver, which are dependent on estrogen (Berry 2003). The reduction of precursors such as phospholipoprotein and energy stores (glycogen) has been shown to influence liver

weight loss (Berry and Brake, 1985) and ovary involution (Berry, 2003). Another hormone that affects ovary regression and subsequently overall body weight loss is prolactin. Prolactin is the hormone responsible for broodiness and rises in concentration as egg laying proceeds (Berry, 2003). Eventually, the level of prolactin reaches high enough levels to inhibit the hypothalamic release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) from the pituitary. Prolactin has also been implicated in the reduction of ovarian steroidogenesis (Berry, 2003), which as mentioned previously, induces ovary regression.

Corticosterone is an adrenal glucocorticoid stress hormone affected by the hypothalamic-pituitary-adrenal (HPA) axis which is activated by the need to mobilize body energy stores (stress). It serves a number of roles during molting such as regulation of behavioral patterns, coping cycles, and general well being (Cheng et al., 2001). In addition, corticosterone can inhibit growth, reduce the size of gonads, increase heterophil/lymphocyte ratio, and reduce antibody response to a specific antigen, as well as increase fear (Littin and Cockrem, 2001). The increase in corticosterone levels are the first significant endocrine changes seen in molted hens and are initiated by stress (Nasir et al., 1999). During a molt, corticosterone levels increase initially and levels decrease as the molt continues. Upon refeeding layer ration at the end of a molt, the levels again increase (Berry, 2003) and then revert to normal levels as the energy level of feed is increased (Webster, 2003). The increased release in corticosterone during molting can cause a decrease in passage rate in molted hens which in turn decreases feed intake (Nasir et al., 1999) and forces the hen to save energy by maintaining metabolic

activity at a low level, which contributes to body weight loss (DeJong et al.,2002). Furthermore, increased corticosterone levels retard spleen weights as immunological organs such as the spleen are sensitive to corticosterone (Post et al., 2003), also contributing to body weight loss.

Heterophil and lymphocyte ratios are frequently used as stress indicators in laying hens during molting (DeJong et al., 2002). They are fairly easily obtained through blood samples from the hen and analyzed by staining slides and enumerating the cells. Studies have shown that during molting there is a positive relationship between heterophil and lymphocytes in the blood and corticosterone levels (DeJong et al., 2002). The increase in heterophil and lymphocyte ratios can be explained in the same manner as the increase in corticosterone levels as corticosterone causes changes in circulating populations of leukocytes (Webster, 2003). Limited feeding has also been shown to increase the ratio (DeJong et al., 2002). The change in circulating populations of leukocytes has been proven to affect the immunological defenses against infection and disease (Webster, 2003).

In addition to ovary regression, another goal of a successful molt is feather loss and replacement, which is believed to be controlled by the thyroid hormones. An increase in thyroid hormones results in an increase in feather loss. While ovary regression and feather loss function under separate control, much interest has arisen about the relationship between ovarian and thyroid steroids (Berry, 2003).

FEED WITHDRAWAL MOLTING

While there are several molting methods, feed withdrawal has been the most popular due to ease of application, low cost, low mortality rates, and agreeable post-molt performance (Keshavarz and Quimby, 2002; Bell, 2003). Some feed withdrawal periods are relatively short, as little as 4 days (Webster, 2003), while some are longer, as long as 14 days (Bell, 2003). Following the feed withdrawal period, the lighting scheme is returned to normal (16 hours light : 8 hours dark) and the hens are fed layer ration and returned to production or a low energy corn- based maintenance diet which allows for a resting period (North and Bell, 1990). A short resting period of 0 to 7 days can result in flocks returning to peak production in as little as 4 weeks; whereas, a longer resting period can last as long as 21 days and results in peak production at 10 to 11 weeks post molt (North and Bell, 1990).

Feed withdrawal (FW) molting methods are seen as logical because wild birds exhibit similar behavior when they undergo a natural molt; they lose as much as 40% of their body weight, half of which is attributed to ovarian regression (Berry, 2003) while refusing food until the later stages of the molt (Mrosovsky and Sherry, 1980). Berry (2003) states that survival with little or no food for relatively long periods of time is a normal feature of a chicken's physiology.

Feed withdrawal molting methods have been shown to alter behavior in force molted hens (Webster, 2003). Some behavioral activities that have been studied include

gakel calls, non-nutritive pecking, preening, head shaking, aggression, and general activity (sitting, standing, walking, etc.). Gakel calls are vocalizations produced and are thought to be signs of frustration in molted hens. Non-nutritive pecking is pecking at objects such as cage wires, the floor, or any other object placed into the birds view. This is a typical response of birds when feed is withdrawn and occurs in response to hunger. Birds are natural foragers and as a result of feed removal, they continue this behavior and search for food in their environment. Preening may be performed as a displacement action in situations of conflict or frustration; however, some researchers have suggested that preening in molted hens is related to integument stimulation as feathers are being pushed out (Webster, 2000). Head shaking is a behavioral action that is thought to be related to coping response to constraint, disturbance, and environmental changes. Head shaking may also be an alerting response, manifested in an attempt to increase arousal (Webster, 2000). Aggression has been shown to increase during periods of feed withdrawal in molted hens. When hens are deprived of feed they tend to not only peck at non-nutritive objects but other hens as well. If one hen is more dominant in the group, she will continue to peck at subordinate hens, possibly until death (Webster, 2000). Overall activity of molted hens also increases over the course of a molt. This is greatly attributed to boredom because feed is removed and birds are frustrated.

Animal welfare concerns have recently affected the means by which commercial producers molt their hens. Efforts have been made to reduce or even eliminate the use of programs that require complete removal of feed from hens. For this reason, alternative methods that do not require complete removal of feed are being considered. Currently, a

variety of feedstuffs are being developed as dietary alternatives to feed withdrawal to alleviate concerns about increased *Salmonella* infection (Holt, 2003), effects of fasting (Berry, 2003), and stress (Post et al., 2003). Birds stressed by feed withdrawal molting show signs of hyperactivity, increased drinking and increased non-nutritive pecking (pecking at non-food substances). These behaviors are characteristic of hunger and frustration (DeJong et al., 2002). In addition to stress associated with feed withdrawal, hens experience an increased susceptibility to *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) infection, marked by increased intestinal shedding and dissemination of SE to internal organs such as the liver, spleen and ovary (Holt and Porter, 1992). Consequently, practices such as feed withdrawal, which increase the susceptibility of SE infection in hens also increase the risk of human salmonellosis from SE contaminated eggs (Holt and Porter, 1992).

SALMONELLA INVASION IN MOLTED HENS

Feed withdrawal molting has been shown to increase stress in hens, which leads to a compromised immune system (Holt, 2003). By compromising the immune system, hens are more susceptible to infection by a number of organisms, in particular *Salmonella enteritidis* (Holt, 1993; Holt, 2003). The indigenous microflora in the alimentary canal naturally provide a hostile environment for infectious agents such as SE; however, during feed withdrawal molting, the environment is altered (Holt and Porter, 1992). The first line of defense in the chicken's alimentary canal is the crop,

which is a non-secretory organ that is populated by lactobacilli with a low pH of 4-5 (Holt, 2003). When feed enters the crop, it is fermented by lactic acid bacteria which in turn decreases the pH and inhibits the growth of enteric pathogen (Hinton et al., 2000). However, during feed withdrawal, microbiological changes occur in the crop due to the absence of feed, thus allowing pH to increase and increasing susceptibility to pathogen infection (Hinton et al., 2000; Durant et al., 1999). The intestines of the chicken are another line of defense. When feed is present as fermentation in the intestines, particularly the ceca, volatile fatty acids (VFA) are produced, which act on enteric pathogens much in the same way lactic acid does in the crop (Hentges, 1983; Russell and Diez-Gonzalez, 1998; Van Der Wielen et al., 2000). Again, without feed in the alimentary canal, enteric bacteria are more able to colonize the intestines. Upon colonization of the intestinal epithelium, SE is able to invade a variety of organs including the spleen, liver, ceca, ovary and oviduct (Berry and Brake, 1985; Gast, 1994).

SE contamination has been reported to occur in two ways: either in-egg by which the eggs are contaminated during the formation in the ovary and oviduct or by on-egg contamination by which eggs are contaminated by fecal contamination of the surface (Barua and Yoshimura, 2004). In both modes of transmission, the hen shows little to no signs of being infected with SE (Guard-Petter, 2001). While in-egg contamination is rarely seen without being associated with shell contamination (0.005% of the time), it is likely to be due to ovarian infection (Gast, 1994). The ovary is the site of egg yolk formation (Burley and Vadehra, 1989); therefore, when the ovary is contaminated, SE is deposited in the yolk (Gast et al., 2004). The yolk does contain antibodies which can

inhibit bacterial growth (Gast, 1994); however at storage temperatures above 20°C, bacteria flourish (Gast and Holt, 2001).

On-egg contamination has been shown to be the primary method of contamination with 7% of eggs being contaminated on the shell (CDC, 2004). Shell contamination occurs after the formation of the egg shell either in the oviduct or by fecal contents as the cloaca is the common orifice for the reproductive and digestive tracts (Takata et al., 2003). While the occurrence of on-egg contamination is also low, it is increased by relatively high humidities, low temperatures during storage (Humphrey, 1994) and cracked shells (Guard-Petter, 2001). Alternative molting methods must be sought to effectively molt hens while maintaining the indigenous microflora in order to reduce colonization or prevent SE infection (Ricke, 2003).

ALTERNATIVE MOLTING DIETS

Historically researchers have examined alternative diets to feed withdrawal that provide similar benefits while not altering the health of the animals. General dietary modification strategies have involved either constructing diets that are deficient in some nutrients such as sodium or contain an excess of a particular compound such as zinc. In the past, studies have been conducted using diets mixed with high zinc concentrations (Bell, 2003), thyroxine (Keshavarz and Quimby, 2002), and low sodium concentrations (Berry and Brake, 1985) to induce molt. Such diets have yielded inconsistent results, are costly and can cause negative behavior such as cannibalistic pecking (Biggs et al., 2004;

Webster, 2003). Low calcium diets have also been used; however, ovary and oviducts did not regress to a non-productive state, production did not cease completely, and the diets have been shown to cause osteoporosis and temporary paralysis (Webster, 2003). A second general approach has incorporated the use of insoluble plant fibers such as grape pomace (Keshavarz and Quimby, 2002), cotton meal (Davis et al., 2002), jojoba meal (Arnouts et al., 1993; Vermaut et al.,1997), wheat middlings (Seo et al., 2001), and alfalfa (Landers et al., 2005a,b; Woodward et al., 2005).

ALFALFA AS AN ALTERNATIVE MOLTING DIET

Alfalfa (*Medicago sativa*) is a readily available feedstuff available in most commercial locations (Landers et al., 2005b). Alfalfa is high in protein (17%), low in energy (1,200 kcal/kg), and relatively high in calcium (1.44%; NRC, 1994; Matsushima, 1972). Alfalfa has been widely used as cattle feed for hundreds of years due to its palatability and nutritional value (Hansen, 1972). Before chickens were confined, many were dependant on pasture with the most satisfactory pasture in the United States being alfalfa (Hansen, 1972). Today, alfalfa is still being used in poultry rations, but in small quantities due to the low energy content.

Alfalfa has been used for many years to add pigment to egg yolks as hens do not produce yolk pigment in their bodies (Madiedo and Sunde, 1964). Xanthophyll which is present at 220 mg/kg (Madiedo et al., 1964; NRC, 1994) in alfalfa is estimated to contribute to 70% of the total yolk color while zeaxanthin contributes the remaining 30%

(Madiedo and Sunde, 1964). Pro-Xan is an alfalfa leaf concentrate made from the wet fraction of fresh alfalfa (Kuzmicky et al., 1977) which includes protein, amino acids and other nutrients that has about 1.7 times the xanthophyll availability when compared to alfalfa meal (Kuzmicky and Kohler, 1977). Kuzmicky et al., (1972) showed that up to 54% Pro-Xan could be fed without weight loss. Alfalfa increases egg quality by adding pigment to the yolk, however, with increased amounts of alfalfa, the occurrence of blood spots also increased, causing eggs to be unmarketable, thus causing losses to producers (Sauter et al., 1965).

Another disadvantage to feeding alfalfa is the negative effects on production rate and fertility and hatchability due to the saponin content in alfalfa (Kingan and Sullivan, 1964). Saponins are naturally occurring sugar conjugates of steroids (Sen et al., 1998) that are found in foods such as soybeans, chick peas, spinach and asparagus, that humans consume daily without harm (Malinow et al., 1981). In humans, saponins have been shown to be beneficial due to hemolytic, anticancer, and anti-inflammatory properties (Huhman and Sumner, 2002). Saponins mainly affect nonruminants as rumen microorganisms are able to degrade saponins (Lu et al., 1987; Lu and Jorgensen, 1987). Saponin content in alfalfa has been estimated to be between 1.5 and 3.3% and growth is inhibited in chickens at 0.1 to 0.5% saponin content (Malinow et al., 1981). This is due to the ability of saponins to alter the palatability (bitterness) thus decreasing feed consumption and / or its influence on digestion and absorption in the gastrointestinal tract (Oleszek, 1996). Udea et al. (2002) suggests that factors other than palatability

affect feed intake and thus growth. Digestion and absorption of nutrients are affected due to the saponins irritating the membranes of the gastrointestinal tract (Oleszek, 1996).

Despite the disadvantages of feeding alfalfa to chicks and broilers, these characteristics of alfalfa make it an ideal molting diet. According to Swanson and Bell (1974) an ideal molting diet should be inexpensive, result in low mortalities, be easy to apply, and lead to post molt production comparable to that of feed withdrawal molt. In addition, a molt diet should cause a cease in egg production to allow for reproductive rejuvenation.

A study was conducted by Woodward et al. (2005) where an alfalfa diet was also shown to successfully induce molt as compared to the traditional feed withdrawal molt. Hens that underwent the alfalfa molt showed greater lactic acid and VFA concentrations than feed withdrawal hens, indicating alfalfa as an inhibitory diet for SE colonization (Woodward et al., 2005). Combining layer ration with alfalfa is also believed to induce molt similarly to alfalfa alone as well as contribute to greater fermentation than feed withdrawal and increase feed intake as compared to 100% alfalfa diets (Moore et al., 2004). Eggs laid by alfalfa molted hens were overall heavier, longer and had higher albumen heights (Landers et al., 2005a). In addition, no differences were found between taste/texture and color of eggs laid by feed deprived and alfalfa molted hens. According to these studies, alfalfa induces molt just as well as feed withdrawal, has the ability to reduce SE colonization (Landers et al., 2005a; Woodward et al., 2005) and shows no detectable differences in consumer preference in eggs from alfalfa molted hens (Landers et al., 2005b).

In addition to using alternative diets to induce molt and potentially reduce SE colonization, researchers have used feed additives to further alleviate concerns of SE infection. SE has been shown to be reduced by the presence of mannose and lactose in the diet (Oyofo et al., 1989); however, the results are variable (Corrier et al., 1990; Hinton et al., 2000). Other carbohydrates such as dextrose, maltose and sucrose had no effect on SE colonization (Oyofo et al., 1989). The addition of prebiotics such as oligosaccharides to poultry diets have also been shown to inhibit SE colonization while beneficially affecting the indigenous microflora (Bailey et al., 1991; Orban et al., 1997; Fernandez et al., 2002). In the following chapters, the potential mechanisms of prebiotics will be discussed.

PREBIOTICS

Prebiotics have been defined by Gibson and Roberfroid (1995) as indigestible food ingredients which stimulate the growth and/or activity of a select number of bacteria in the colon and improve the host's health. In order for a food ingredient to be considered a prebiotic, it must have certain characteristics (Table II-1). Prebiotics have been shown to alter gastrointestinal microflora, alter the immune system, prevent colonic cancer, reduce pathogen invasion including pathogens such as SE and *E.*coli and reduce cholesterol and odor compounds (Cummings at al., 2001; Cummings and Macfarlane, 2002; Patterson and Burkholder, 2003).

Table II-1. Characteristics for food ingredients to be considered a prebiotic[1]

Be neither hydrolyzed or absorbed in the upper gastrointestinal tract
Selectively enrich one or a limited number of beneficial bacteria to grow and/or be metabolically activated
Beneficially alter colonic flora and their activities in the host
Beneficially effect luminal or systemic aspects of the host

[1] Adapted from Gibson and Roberfroid, 1995 and Patterson and Burkholder, 2003

Prebiotics are short-chain carbohydrates that are indigestible by human, animal and poultry digestive systems. The major effects of prebiotics have been reviewed by Cummings and Macfarlane (2002) and include: production of short-chain fatty acids and lactate, selective increases in *bifidobacteria* and *lactobacilli*, increase in pathogen resistance and improved calcium and magnesium absorption. Once prebiotics reach the cecum, they are most effective (Cummings et al., 2001).

The gastrointestinal tract of poultry has been studied at a great extent (Van Der Wielen et al., 2000, Salanitro et al., 1974, Apajalahti et al., 2004) and has proven to be a remarkable physiological structure. The gastrointestinal tract includes the structures of the digestive tract, which are responsible for nutrient and water absorption, fermentation and waste excretion. Within these structures is a diverse, complex microbial ecosystem with the majority of bacteria residing in the cecum (Salanitro et al,. 1974). The cecum in birds is much different as compared to mammalian ceca, due to the increased surface area, which is helpful in hydrolysis, absorption, and fermentation (Vispo and Karasov,

1997). Most of the bacteria in the cecum are considered anaerobic and include species such as *Lactobacilli, Bifidobacterium* and *Propioniobacterium* (Salanitro et al., 1974). The microflora in the ceca work together to maintain a stable ecosystem in order to form a natural resistance to infections produced by enteric pathogens (Hentges, 1983); this is accomplished by forming a physical barrier to keep intestinal bacteria in check and protect against enteric pathogens by discriminating between enteric and resident microflora (Lu and Walker, 2001). Enteric pathogens possess specialized processes, which allow them to penetrate the intestinal epithelium. Inside the intestinal epithelium the pathogen can adhere to the surface, colonize and establish permanent residence, which can cause disease if not prevented by the natural microflora (Lu and Walker, 2001). While the indigenous microflora flourish in the presence of prebiotics, enteric pathogens such as *Escherichia coli*, *Clostridium perfringens* (Cummings and Macfarlane, 2002) and *Salmonella* (Bailey et al., 1991) are inhibited by them. These pathogens are inhibited due to the fact that they are unable to use prebiotics as a sole carbon energy source and when fermentation by indigenous microflora increases in the presence of prebiotics so do volatile fatty acid concentrations, which decrease the pH to levels intolerable by many pathogenic bacteria (Cummings and Macfarlane, 2002). In addition increased activity by the microflora inhibits attachment of bacteria, which is essential for infection (Flickinger et al., 2003).

The use of prebiotics in human as well as animal diets is a generally new concept in the United States. In Japan, prebiotics are a normal ingredient in many diets, especially weaning piglets, and the use is ever increasing in Europe (Flickinger et al.,

2003). Common prebiotics currently available for human and animal consumption include: isomaltooligosaccharides, Oligomates, Palatinoses, Polydextrose, Raftilines, Soybean oligosaccharides, xylooligosaccharides and the most popular being fructooligosaccharides.

FRUCTOOLIGOSACCHARIDES (FOS)

The most commonly used prebiotic in both human as well as animal diets is fructooligosaccharide (FOS), which is a naturally occurring oligosaccharide usually of plant origin and is the only product recognized and used as a colonic food ingredient and prebiotic (Bomba et al., 2002; Gibson and Roberfroid, 1995). FOS is composed of one molecule of glucose and one to three molecules of fructose (Bengmark, 1998) and can be marketed commercially as Raftilose or Nutraflora or can be synthesized from food sources (Kaplan and Hutkins, 2000). Common foods which contain fructooligosaccharides are garlic, onion, artichoke and asparagus (Gibson and Roberfroid, 1995) and common animal feed ingredients which contain FOS include alfalfa meal, barley, peanut hulls, wheat middlings and wheat bran (Flickinger et al., 2003).

Due to the β-linkages possessed by FOS, it is able to resist enzymatic degradation and absorption in the upper gastrointestinal tract to reach the cecum where the majority of fermentation occurs in chickens (Gibson and Roberfroid, 1995; Juskiewicz et al., 2004; Xu et al., 2003). Once in the ceca, FOS is selectively fermented

by strains of bifidobacteria subsequently decreasing the pH. The decrease in pH is attributed to the production of VFA's by bifidobacteria, mainly acetate, lactate (Gibson and Roberfroid, 1995) and butyrate (LeBlay et al., 1999). While this is the primary method of pathogen control, Gibson and Roberfroid (1995) report this may not be the only method. When compared to glucose as a growth promotant for bifidobacteria as well as lactobacillus, FOS proved to be a comparable substrate (Kaplan and Hutkins, 2000).

The level of FOS to include in diets has been a topic of interest for years. Bailey et al. (1991) reported that 0.375% FOS was not enough to inhibit SE colonization, however, 0.75% was sufficient to inhibit SE colonization. The results from Waldroup et al. (1993) showed similar results, concluding that there were no effects of 0.375% FOS on broiler body weight, feed efficiency, dressing percentage or abdominal fat, however, Ammerman et al. (1988) reported conflicting results with 0.375% FOS producing heavier birds and improving feed efficiency. Wu et al. (1999) found the optimal level of FOS in chicken diets to be between 2.5 and 5%. These results were supported by Xu et al. (2003) which found 4% FOS to improve growth, while higher levels of 8% produced poorer results. Feeding higher levels of FOS (>20%) has been shown to cause flatulence and loose stools (Flickinger et al., 2003). In addition to adding FOS to feed, it has also been added to water at a level of 2% (Janssens et al., 2004). This study showed that at 2%, there were no effects on Salmonella typhimurium excretion and water intake increased markedly.

RESEARCH OBJECTIVES

The purpose of this research was to determine if alfalfa was a valid molt diet as an alternative to feed withdrawal and if the addition of a fructooligosaccharide prebiotic to the alfalfa molt diet further decreased *Salmonella enteritidis* colonization of organs of laying hens. Molting is a common industry practice in which laying hens, after a resting period, return to increased egg production and quality. Animal welfare concerns have recently encouraged producers to develop alternative molting methods. A study was conducted to determine if alfalfa combined with layer ration, at levels of 100, 90, 80, and 70% alfalfa, was a feasible alternative to feed withdrawal molting.

During a molt, the gastrointestinal tract of a hen is altered. Volatile fatty acid concentrations have been shown to decrease as well as lactic acid concentrations. A decrease in these acids by the indigenous microflora indicates a decrease in fermentation. To determine if alfalfa combined with a fructooligosaccharide prebiotic could positively contribute to the indigenous microflora and fermentation, a study was conducted *in vitro* using laying hen cecal bacteria as an inoculum with alfalfa and layer ration feed substrates being compared as the primary dietary sources.

Consequently during a molt, when VFA and lactic acid production is decreased, pathogen invasion is increased. One of the most important pathogens in poultry research is *Salmonella* due to contamination of poultry meat and eggs which is passed on to the consumer. Another *in vitro* study was designed and conducted to evaluate the effects of

alfalfa and layer ration combined with FOS on *Salmonella* growth in laying hen cecal bacteria after fermentation time had been allowed.

While *in vitro* studies are helpful in determining the abilities of alfalfa and FOS to ferment and produce VFA's and lactic acid and inhibit *Salmonella*, an *in vivo* study was essential for bringing all the information together. Therefore, laying hens were inoculated with SE and fed diets containing 90% alfalfa and 10% layer ration then FOS was added at two levels (0.75 and 0.375%) as the optimum level of FOS to be added to a poultry diet is still unclear. This study evaluated the effects of alfalfa and FOS on SE colonization in organs, as well as the effects of different levels of FOS in the diet.

CHAPTER III

UTILIZING DIFFERENT RATIOS OF ALFALFA AND LAYER RATION FOR MOLT INDUCTION AND PERFORMANCE IN COMMERCIAL LAYING HENS*

SYNOPSIS

Molting is a common practice used by the commercial egg industry to rejuvenate flocks for a second or third laying cycle. During this time the hens rest from production and the reproductive organs are rejuvenated in order to increase production and quality in the next laying cycle. While feed withdrawal is the most popular and effective method of molt induction, it has come under scrutiny due to food safety issues and animal welfare issues. This study involved feeding alfalfa mixed with layer ration at different ratios to hens to determine their ability to induce molt. The treatment ratios were 100% alfalfa (A100), 90% alfalfa/10% layer ration (A90) and 70% alfalfa/30% layer ration (A70). In addition, a full fed (FF) nonmolted control and a feed withdrawal (FW) negative control were used. Alfalfa is an insoluble, high fiber feedstuff with low metabolizable energy. Egg production for A90 and feed withdrawal (FW) treatments

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ceased completely by d 6 while birds fed A100 and A70 ceased by d 8. Ovary and oviduct weight of hens fed all molting diets decreased significantly ($P< 0.05$), by an average of 1.5-2.5 % (body weight basis), compared to FF control during the 9-d molt induction period. As % layer ration increased, feed intake also increased and % body weight loss decreased during the 9-d molt induction period. Hens molted by FW lost an average of 25.8% body weight whereas A70 hens lost 18.9% body weight. Non molted hens (FF) and A70 treatment hens had significantly lower ($P<0.05$) egg production when compared to all other treatments over the 39 wk post molt period. FF treatment hens also had significantly lower ($P<0.05$) albumen heights when compared to all other treatments. From these results, alfalfa or alfalfa mixed with layer ration appears to be viable alternatives to conventional feed withdrawal methods for the successful induction of molt and retention of postmolt performance.

INTRODUCTION

The commercial egg industry commonly uses induced molt procedures to rejuvenate flocks for a second or third laying cycle and to increase profits. According to Bell (2003), approximately 75% of commercial laying facilities in the United States used an induced molt program in order to rejuvenate flocks for increased productivity. Implementing an induced molt program can result in a 30% higher profit margin for producers when compared to an all-pullet operation (Bell, 2003). In addition to increased profit margins, an induced molt rejuvenates the hens reproductive tract to

produce higher quality eggs which are more marketable (Keshavarz and Quimby, 2002). The main purpose of molting is to cease egg production in order for the hens to enter a non-reproductive state which increases egg production and egg quality post molt (Webster, 2003).

While there are several molting methods, feed withdrawal has been the most popular due to ease of application, economic benefits, and agreeable post-molt performance (Keshavarz and Quimby, 2002; Bell, 2003). Feed withdrawal (FW) molting methods are seen as logical because wild birds exhibit similar behavior when they undergo a natural molt; they lose as much as 40% of their body weight while refusing food until the later stages of the molt (Mrosovsky and Sherry, 1980). However, recent concerns have been raised about animal welfare during the feed withdrawal period because it is thought to be harmful to the hens (Webster, 2003). Efforts have been made to reduce or even eliminate the use of such programs that require complete removal of feed from hens. For this reason, alternative methods that do not require complete removal of feed are being considered. Historically researchers have examined alternative diets to FW that provide similar benefits while not altering the health of the animals. General dietary modification strategies have involved either constructing diets that are deficient in some nutrients such as sodium or contain an excess of a particular compound such as zinc. In the past, studies have been conducted using diets mixed with high zinc concentrations (Bell, 2003), thyroxine (Keshavarz and Quimby, 2002), and low sodium concentrations (Berry and Brake, 1985) to induce molt. However, such diets have yielded inconsistent results, are costly and can cause negative behavior such

as cannibalistic pecking (Biggs et al., 2004; Webster, 2003). Low calcium diets have also been used, however, ovary and oviducts did not regress to a non-productive state, production did not cease completely and has been shown to cause osteoporosis and temporary paralysis (Webster, 2003). A second general approach has incorporated the use of insoluble plant fibers such as grape pomace (Keshavarz and Quimby, 2002), cotton meal (Davis et al., 2002), jojoba meal (Arnouts et al., 1993; Vermaut et al.,1997),wheat middlings (Seo et al., 2001), and alfalfa (Kwon et al., 2001; Landers et al., 2004).

Alfalfa is a readily available, high protein, high fiber feedstuff with one of the slowest rates of passage through the avian system (Matsushima, 1972; Sibbald, 1979; Garcia et al., 2000). Alfalfa is well balanced in amino acids, and rich in vitamins, carotenoids and xanthophylls that give poultry carcasses their desirable yellow color (Sen et al., 1998; Ponte et al., 2004). Alfalfa also contains high levels (2-3% of DM) of saponins, which have been shown to have hypocholesterolemic, anticarcinogenic, anti-inflammatory, and antioxidant properties (Klita et al., 1996; Ponte et al., 2004). Alfalfa is extremely advantageous due to the fermentation properties by cecal microflora that are capable of limiting the *in vitro* growth of *Salmonella* Typhimurium when alfalfa is present (Donalson et al., 2004a,b). The objective of this study was to evaluate the effectiveness of different ratios of alfalfa combined with layer ration on the induction of a molt, post-molt production and post-molt egg quality (up to and including wk 39).

MATERIALS AND METHODS

Molting Procedure

A total of 120 White Single Comb Leghorn (SCWL) laying hens 70-80 wk of age were obtained from a commercial laying facility. Birds were housed 1 per cage at the Texas A&M University (TAMU) Poultry Science Research Center in College Station, Texas and allowed three wk for acclimation. During this time the birds were fed a complete layer ration (Table III-1; Table III-2) *ad libitum* and allowed full access to water. Egg production was monitored to insure all hens were healthy and actively producing. After acclimation, hens were moved to a nearby house and placed 2 birds per cage for the molting procedure. The hens were then divided into five treatment groups with 24 birds per treatment: nonmolted control- full fed (FF), negative control- feed withdrawal (FW), 100% alfalfa (A100), 90% alfalfa / 10% layer ration (A90), and 70% alfalfa / 30% layer ration (A70; Table III-2). All treatments were allowed *ad libitum* access to water and their respective diets. Hens were placed on an artificial lighting program of 8-hour light: 16-hour dark for one wk prior to molt to allow for normal production to continue while hens were photosensitized to ensure a more complete and rapid molt (Andrews et al., 1987a). Treatments were randomly assigned to cages throughout the house to ensure there was no variability in egg production or reproductive tract regression due to light stimulation. Hens were then molted for 9 d (Kwon et al.,

Table III-1. Composition of Texas A&M University (TAMU) layer ration and alfalfa-layer ration combination molt diets

Ingredient	TAMU layer ration [a] (FF)	A90[b]	A70[b]	A100[b]
	----------(g/kg) ----------			
Corn, yellow	567.18	56.72	170.15	----[e]
Soybean meal	316.33	31.63	94.90	----[e]
Vegetable oil	76.82	7.68	23.05	----[e]
Mono calcium phosphate	16.86	1.69	5.06	----[e]
Calcium carbonate	15.62	1.56	4.69	----[e]
Methionine, 98%	1.69	0 .17	0.51	----[e]
Vitamin premix[c]	2.50	0 .25	0.75	----[e]
NaCl	2.50	0 .25	0.75	----[e]
Trace mineral premix[d]	0.50	0 .05	0.15	----[e]
Alfalfa	----[e]	900.00	700.00	1,000.00
Total	1,000.00	1,000.00	1,000.00	1,000.00

[a]For diet formulation, crude fat concentrations were fixed at 100 g/kg

[b] A90=90%alfalfa/10% layer ration; A70=70% alfalfa/30% layer ration; A100=100% alfalfa

[c]Provides mg/kg of diet unless otherwise noted: vitamin A, 8,818 IU; vitamin D, 2,205 IU; vitamin E, 5.86 IU; vitamin K, 2.2 IU; thiamine, 1.1 IU; riboflavin, 4.4 IU; niacin, 22 IU; pantothenic acid; choline, 500 IU; vitamin B_{12}, 0.013 IU; biotin, 0.055 IU.

[d]Trace mineral premix (Nutrius Premix Division, Bioproducts Inc., Cleveland, OH), provided as milligrams per kilogram of diet unless otherwise noted: Mn, 68.2; Zn, 55; Cu, 4.4; I, 1.1; Se, 0.1.

[e] None used

Table III-2. Composition Analysis of Texas A&M University (TAMU) layer ration and alfalfa-layer ration combination molt diets

Nutrient Name	TAMU Layer Ration (FF)	A90[a]	A70[a]	A100[a]
Dry Matter (%)	90.10	91.810	91.430	92.00
Crude Protein (%)	15.00	17.250	16.750	17.50
Ether Extract (%)	2.93	2.793	2.979	3.00
Crude Fiber (%)	2.30	21.920	17.560	24.10
Ash (%)	10.15	9.115	9.345	9.00
Calcium (%)	3.25	1.621	1.983	1.44
Total Phosphorus (%)	0.47	0.245	0.295	0.22
Avail. Phosphorus (%)	0.25	0.223	0.229	0.22
Metabolizable Energy (per kg)	2,872	962.2	1,386.6	750
Total ME (per kg)	2,965	1,206	1,597.2	1,011
Xanthophyll (mg/kg)	12.32	91.232	73.696	100.00
Methionine (%)	0.31	0.247	0.261	0.24
Cystine (%)	0.27	0.198	0.214	0.19
Lysine (%)	0.72	0.729	0.727	0.73
Arginine (%)	0.93	0.714	0.762	0.69
Threonine (%)	0.56	0.677	0.651	0.69
Tryptophan (%)	0.17	0.224	0.212	0.23
Glycine (%)	0.61	0.799	0.757	0.82
Serine (%)	0.72	0.720	0.720	0.72
Histidine (%)	0.40	0.553	0.519	0.57
Isoleucine (%)	0.59	0.662	0.646	0.67
Leucine (%)	1.40	1.211	1.253	1.19
Valine (%)	0.69	0.825	0.795	0.84
Phenylalanine (%)	0.70	0.799	0.777	0.81
Tyrosine (%)	0.57	0.786	0.738	0.81
Choline (mg/kg)	1,314	1,392.3	1,374.9	1,401
Linoleic acid (%)	1.88	0.611	0.893	0.47
Sodium (%)	0.13	0.094	0.102	0.09

[a] FF= Full Fed (non molted); A90=90%alfalfa/10% layer ration; A70=70% alfalfa/30% layer ration; A100=100% alfalfa

2001; Landers et al., 2004) as part of a rapid molt as described by North and Bell (1990) and Parkhurst and Mountney (1988).

During the molt, bird weights were monitored at d 1, 5, 7, and 9. In accordance with the Texas A&M University Lab Animal Care Committee (ULACC) animal use protocols, any hen reaching 25% weight loss prior to the end of the trial (d 9) were removed from their respective diet and immediately placed on a full fed layer ration feeding program. Feed intake was measured by weighing each diet prior to the start of the molt and after the nine d molt period.

Collection of Organs, Egg Production and Quality Parameters

At the end of the molt, 60 birds were euthanized with CO_2 gas according to approved Texas A&M ULACC protocols and the ovaries, oviducts, kidneys, hearts, livers, and spleens were excised aseptically and weighed and expressed as relative weights (% of body weight). The remaining 60 birds were returned to TAMU layer ration on an *ad libitum* basis (North and Bell, 1990; Parkhurst and Mountney, 1988). The lighting program was changed to 16-hour light: 8-hour dark to stimulate egg production. Egg production was measured daily (% hen-d assuming 1 egg per d = 100%) while egg quality parameters were measured twice a wk. Egg weight was measured using a balance[1] and recorded to the nearest 0.01g. Egg length, albumen height, yolk height and yolk diameter were measured using a caliper and recorded to the

[1] Navigator model N14120, Ohaus Corporation, Pinebrook, NJ

nearest 0.1 mm. Shell thickness was evaluated using NaCl solutions (Keshavarz and Quimby, 2002), the specific gravity of which ranged from 1.065 to 1.090 in increments of 0.005. Shell strength (kg) was measured using an Instron Universal Testing Machine[2] with a 50 kg-load cell at a 10 kg-load range and a crosshead speed of 50 mm/minute (Park et al., 2004). Haugh units (HU) were calculated taking into account egg length and albumen height as an indicator of interior egg quality (Silversides et al., 1993). Egg production and quality were measured for 39 wk after molting.

Statistical Analysis

Data were analyzed using the general linear models procedure of SAS software (2001). Differences in parameters (egg production, feed intake, grams body weight loss, % body weight loss, organ weights, internal egg quality, external egg quality) among treatment groups, when significant, were compared using Duncan's multiple range test. None of the data was transformed prior to analysis. Level of significance used in all results was $P<0.05$.

[2] Model 1011, Instron Corp., Canton, MA

Table III-3. Effect of alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet on feed intake, body weight loss, and percentage body weight loss during the 9-d molting period

Treatment[1]	Feed intake	body weight loss	body weight loss
	(g/bird)	(g/bird)	(%)
FF	736.4 ± 16.5[a]	82.2 ± 24.7 [c]	5.2 ± 1.5[c]
FW	NA	400.9 ± 11.4[a]	25.8 ± 0.6[a]
A100	82.0 ± 22.6[d]	392.4 ± 9.9[a]	25.1 ± 0.5[a]
A90	272.3 ± 39.0[c]	373.3 ± 10.8[a]	23.9 ± 0.6[a]
A70	409.4 ± 23.5[b]	289.2 ± 13.0[b]	18.9 ± 0.7[b]

[a-d] Means within a column with no common superscripts differ significantly ($P < 0.05$).n=6, 24, and 24 for feed intake, body weight loss, and % body weight loss, respectively.

[1]FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.

RESULTS AND DISCUSSION

Body Mass

Hens fed diets A100, A90 and FW showed significantly greater ($P<0.05$) percent body mass loss (25.1%, 23.9% and 25.8% respectively) than those fed the A70 diet (18.9%). FF birds exhibited the least amount of percent body mass loss (5.2%) when compared to all other treatments of molted hens (Table III-3). Body mass loss has been shown to be directly related to post molt performance. In order to optimize post molt performance, a body mass loss of 25-30% should be achieved (Baker et al., 1983). Approximately 25% of the body mass lost was attributed to a decrease in liver and reproductive organ weights (Berry and Brake, 1985). The weight loss exhibited by non molted (FF) hens can be explained by the reduced photoperiod as photoperiod and nutrient deprivation have similar modes of action on the hypothalamic hypophyseal axis causing an inhibition of circulating reproductive hormone concentrations with subsequent ovary regression and weight loss (Andrews et al., 1987a; Berry, 2003). The reduced photoperiod also leaves fewer daylight hours for feeding which decreases feed consumption and causes weight loss as exhibited by all hens (Andrews et al., 1987b). A100 and A90 hens lost more body mass than A70 hens due to a decreased feed intake which can be attributed to a number of factors including a higher percentage of alfalfa in the diet.

Table III-4. Effect of alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet on post molt organ weights (as % body weight basis) [1]

Treatment[2]	ovary	oviduct	intestine	kidney	heart	liver	spleen
	---------- (%) ----------						
FF	2.17 ± 0.21[a]	3.98 ± 0.30[a]	3.52 ± 0.20[a]	0.43 ± 0.02[a]	0.48 ± 0.01	2.25 ± 0.05[a]	0.09 ± 0.006
FW	0.55 ± 0.07[b]	1.53 ± 0.07[b]	2.71 ± 0.12[b]	0.35 ± 0.01[b]	0.46 ± 0.01	1.49 ± 0.03[d]	0.11 ± 0.007
A100	0.71 ± 0.09[b]	1.73 ± 0.08[b]	2.85 ± 0.09[b]	0.37 ± 0.02[b]	0.46 ± 0.01	1.60 ± 0.05[cd]	0.10 ± 0.005
A90	0.60 ± 0.04[b]	1.77 ± 0.06[b]	3.11 ± 0.12[ab]	0.39 ± 0.01[b]	0.46 ± 0.01	1.69 ± 0.05[bc]	0.10 ± 0.005
A70	0.48 ± 0.5[b]	1.69 ± 0.14[b]	3.46 ± 0.12[a]	0.45 ± 0.05[a]	0.46 ± 0.01	1.80 ± 0.08[b]	0.11 ± 0.008

[a-d] Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1] Relative organ weight (%) = (organ weight/100g of body weight) x 100
[2]FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.

Organ Weight

Ovarian weight loss occurs simultaneously with body mass loss due to the regression of the ovaries that is directly associated with the rejuvenation process (Brake, 1993). Unmolted control, full fed hens (FF) had significantly higher ($P<0.05$) ovarian weights than hens in all the other molted treatments (2.17% body weight). No significant differences in ovarian weights were found between FW (0.55 % body weight), A100 (0.71% body weight), A90 (0.60% body weight) or A70 (0.48% body weight; Table III-4) treatments. Similar results were shown by Landers et al. (2005b) where ovarian weights from hens fed 100% alfalfa meal were not significantly different from FW hens. No significant differences ($P<0.05$) were found between treatments when comparing heart and spleen weights. Control (FF) birds had significantly higher liver weights when compared to all other treatments (2.25% body weight) whereas FW treated birds had significantly lower liver weights (1.49% body weight) than all treatments except the A100 group (1.60% body weight). Liver weight loss indicates a loss of liver energy sources such as glycogen and lipids, which are metabolized in the liver (Berry and Brake, 1985). Weight loss in the liver is also indicative of the loss of estrogen-dependent egg component synthesis which is dependent on stimulation from ovarian steroids (Berry and Brake, 1985). The most common ovarian steroids are the estrogens whose target organ is the liver where yolk phospholipoprotein synthesis occurs and is dependent primarily on estrogens (Berry and Brake, 1985). With a higher energy concentration due to increased percentage of layer ration, A70 treated hens were

apparently able to retain liver functionality more like that of full fed birds than were birds fed other alfalfa dietary combinations which are significantly lower in energy. This increase in energy density availability for the A70 birds would explain their increased liver weights.

Feed Intake

All treatments exhibited significant differences ($P<0.05$) in feed intake during the molt. FF treated birds exhibited the greatest feed intake (736.4 g/bird over the 9 d molt) while A70 and A90 treated birds ingested 409.4 g/bird and 272.4 g/bird respectively (Table III-3). A100 treated birds exhibited the least feed intake (82 g/bird). The reduction in feed intake could be due to several factors. These may include appetite suppression in conjunction with the natural molting process (Mrosovsky and Sherry, 1980), low palatability of alfalfa by hens (Sen et al., 1998), or decreased feeding stimulation with reduced daylight hours (Andrews et al., 1987b). Furthermore, alfalfa contains saponins, which may be a factor in the suppression of feed intake and growth (Matsushima, 1972). The slow passage rate of alfalfa may also influence feed intake by giving the hen a feeling of satiety and thus causing them to refrain from eating (Sibbald, 1979). Ueda (2002) suggested that the decreased feed intake is due to the delayed crop emptying. Increasing percentages of alfalfa in the diet tended to decrease feed consumption, as feed consumption in A100 treated hens was significantly lower than A90 and A70 treated hens. This trend suggests that the more diluted the diets are with

layer ration, the higher feed consumption will be. Feed intake was also measured for three wk following the molt. No significant differences were seen between any treatments the first two wk after the molt. However, three wk after the molt FF hens exhibited significantly lower feed intake when compared to all other treatments (data not shown).

Interior and Exterior Egg Quality

Interior and exterior egg quality was examined in this study to determine if the different levels of alfalfa would alter postmolt quality of eggs. Significant treatment differences ($P<0.05$) were identified for external parameters including egg weight, egg length, specific gravity (which indicates shell thickness) and shell breakage strength (Table III-5). Egg weights and lengths were significantly higher for FW, A90 and A70 treatments when compared to FF and A100 treatments. This does not agree with results reported previously by Landers et al. (2005b) where egg weights from hens molted by A100 were not significantly different from FW hens. Specific gravity and shell breakage showed significant differences with treatments FW and A90 significantly higher than all other treatments. Higher specific gravity values are related to thicker egg shells which is a desirable characteristic for the egg industry (Keshavarz and Quimby, 2002; DeKetelaere et al, 2002.). Mabe et al. (2003) reported that 80 to 90% of eggs that ended up being down graded were due to cracked or broken eggshells. These defects result in a loss in profits for the producer and can affect consumer safety, as egg shells are a barrier

Table III- 5. Effect of alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet on external egg quality post molt[1]

Treatment[2]	Weight	Length	Specific Gravity	Shell Breakage
	(g)	(mm)		(kg)
FF	67.78 ± 0.30[b]	60.46 ± 0.24[b]	1.076 ± 0.00[b]	2.97 ± 0.07[b]
FW	70.05 ± 0.35[a]	61.09 ± 0.13[a]	1.077 ± 0.00[a]	2.98 ± 0.05[b]
A100	67.74 ± 0.21[b]	60.28 ± 0.11[b]	1.076 ± 0.00[b]	2.94 ± 0.06[b]
A90	70.68 ± 0.41[a]	61.26 ± 0.14[a]	1.078 ± 0.00[a]	3.22 ± 0.05[a]
A70	70.78 ± 0.42[a]	61.13 ± 0.15[a]	1.076 ± 0.00[b]	2.97 ± 0.07[b]

[a-b] Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1] Means of wk 3-39 postmolt
[2]FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.

Table III-6. Effect of alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet on internal egg quality post molt (wk 3-39) [1]

Treatment[2]	Yolk Diameter	Yolk Height	Albumen Height	Haugh Units
	(mm)	(mm)	(mm)	(HU)
FF	41.78 ± 0.11[ab]	18.17 ± 0.08[c]	7.01 ± 0.14[c]	77.89±3.24[b]
FW	41.48 ± 0.15[b]	18.88 ± 0.07[a]	8.57 ± 0.10[a]	87.11±1.95[a]
A100	41.73 ± 0.11[ab]	18.53 ± 0.08[b]	7.79 ± 0.10[b]	84.27±2.14[a]
A90	41.91 ± 0.16[a]	18.31 ± 0.07[bc]	7.60 ± 0.11[b]	85.08±1.68[a]
A70	41.57 ± 0.10[ab]	18.97 ± 0.08[a]	8.31 ± 0.11[a]	85.02±1.93[a]

[a-c] Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1] Means of wks 3-39 postmolt
[2]FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.

to microorganisms such as *Salmonella* (Mabe et al., 2003). It would appear that the A90 treatment represents the optimal dietary mixture to minimize shell breakage.

Interior quality parameters such as yolk diameter, yolk height, albumen height, and Haugh units also proved to be significantly different due to treatment (Table III-6). Yolk diameters of A90 hens were significantly ($P<0.05$) higher than those of FW hens. Yolk heights were significantly higher for A70 and FW treated hens when compared to those of FF treated hens. FF albumen heights were significantly lower when compared to all other treatments indicating a decrease in internal egg quality. Similar results were seen when grape pomace was used as an alternative to feed deprivation for the induction of molt (Keshavarz and Quimby, 2002). Landers et al. (2005b) reported albumen heights significantly lower than current study results; the difference can be explained by a longer post molt period (39 wk) in the current study and a shorter 12-wk post molt period in Landers et al. (2005b). Haugh units were significantly lower for FF treated hens when compared to all molt treatments. These measurements (HU) were comparable to those found in Silversides et al. (1993). Interior quality decreases as hen age increases, however, after a complete molt egg quality is equivalent to that of a 10- to 12-month old pullet (Bell, 1987). When quality increases, more eggs are saleable; this increases profits for producers and keeps supply equivalent to customer demand thus maintaining reasonable prices for consumers (McDaniel and Aske, 2000).

Egg Production and Date of Reentry

On average nonmolted hens fed a layer ration (60.94%) and A70 treatment hens (61.14%) had significantly lower ($P<0.05$) egg production when compared to all other treatments after 39 wk post molt (Table III-7). FW hens (74.29%) not surprisingly had significantly higher egg production that all treatments except A90 treatment hens (73.08%). A100 treatment hens (69.53%) exhibited significantly lower post molt egg production than FW (74.29%) but were not significantly different from A90 hens with 69.53% post molt production. Overall egg production from the pre-molt acclimation period to 39 wk post molt is shown in Figure III-1. The lower egg production rate of the A70 hens after 39 wk post molt is most likely due to an incomplete molt, which is an effect of the high-energy concentration present in the diet in conjunction with its relatively higher layer ration percentage. Other alfalfa diets, especially A90 prove to be comparable to the FW treatment when post molt egg production is concerned. The goal of a viable molting program is to increase post molt egg production and quality. After the molting period, hens improve their egg production due to the rejuvenation of the reproductive organs and overall body weight loss (Alodan and Mashaly, 1999). Increased egg production can relate to profits for the industry depending on bird prices, feed prices and egg demand (Bar et al., 2001). A change in supply as small as 1% can result in a 6% opposite change in egg prices which can cost or make a producer with a typical operation $1.46 million annually (McDaniel and Aske, 2000).

There were no significant differences found between any treatments when d to first egg, days to tenth egg and days between the first and tenth egg were measured. In addition, no significant differences were found between any treatments when examining days to return to 50 to 60% egg production (Table III-8). Hens molted by A100 returned to production 14.8 d after molt induction which is consistent with the observation by Landers et al. (2005b) who also reported that hens fed alfalfa meal during molt return to production 14 d after induction. There were significant differences found from the start of the molt to the first day out of production. On average A70 treated hens took significantly longer (5.75 d) to cease production than FW birds (4.42 d). A100 hens (5.25 d) and A90 hens (4.92 d) were not significantly different from either FW or A70 hens. The sooner hens enter the rest period and cease production, the quicker they will return to production and reach their peak production which occurs within a month of the molting period (North and Bell, 1990). The peak production of a hen during the second cycle after being molted at 65 wk is 75-85%, which is equivalent to a 40-50 wk old flock (Bell, 2003).

Table III-7. Average percent hen-day egg production after induced molt of hens on alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet (wk 1-39).

Treatment[1]	Average Egg Production[2]
FF	60.94 ± 1.55[c]
FW	74.29 ± 1.31[a]
A100	69.53 ± 1.42[b]
A90	73.08 ± 1.26[ab]
A70	61.14 ± 1.48[c]

[a-c] Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.
[2]Egg production was measured daily and 100% represents 1 egg per day

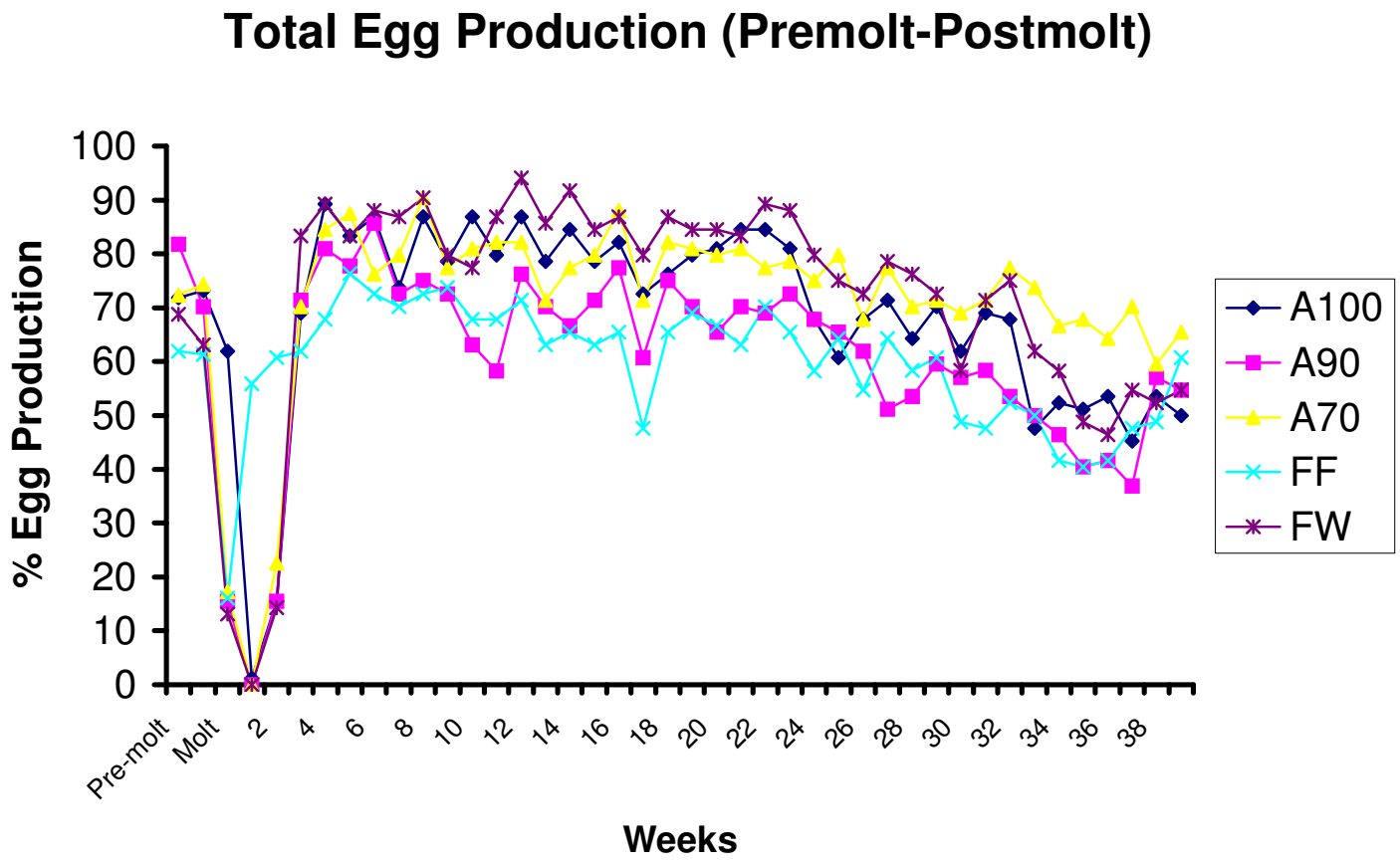

Figure III-1. Percent hen-day egg production by 5 treatments on a wkly basis both during the molt and post-molt of hens on treatments: FF = full feed; FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.
Egg production was measured daily and 100% represents 1 egg per day.

Table III-8. Effect of alfalfa, alfalfa-layer ration, feed withdrawal molt diets and a nonmolt diet on egg production parameters during and after molting

Parameter	FW[1]	A100[1]	A90[1]	A70[1]
1st day out of production from start of treatments	4.42 ± 0.48[b]	5.25 ± 0.28[ab]	4.92 ± 0.15[ab]	5.75 ± 0.33[a]
Days to 1st egg postmolt	15.2 ± 0.44	14.8 ± 0.95	15.8 ± 1.64	14.5± 0.60
Days to 10th egg postmolt	25.6 ± 0.48	26.4 ± 0.77	27.8 ± 2.50	27.8 ± 1.41
Days from 1st to 10th egg	10.4 ± 0.19	11.7 ± 0.38	11.9 ± 0.94	13.3 ± 1.74
Days to return to 50 to 60% egg production	15	16	15	15

[a-b] Means within a row with no common superscript differ significantly ($P < 0.05$). n= 12.
[1]FW = feed withdrawal; A100 = 100% alfalfa; A90 = 90% alfalfa + 10% layer diet; A70 = 70% alfalfa + 30% layer diet.

SUMMARY

Using alfalfa mixed with layer ration as an alternative method for molt induction proved to be effective in molt induction, increasing postmolt egg quality and postmolt egg production when compared to conventional feed withdrawal methods. Alfalfa induced molting offers advantages in that it is readily available across the United States as a common feed for dairy cattle and horses. Furthermore, its *in vitro* fermentability is comparable with other feeds that have been shown to inhibit the growth of enteric pathogens such as *Salmonella* Enteritidis. As animal welfare concerns rise, the industry will continue to seek alternatives to feed withdrawal. Molt diets consisting of alfalfa mixed with layer ration will need to be further investigated to determine the best ratio for molt induction and performance. Based on the results of this study A100 and A90 appear to be the best alternative to feed withdrawal molting methods yielding comparable results. The A70 treatment may also be a viable alternative, however, molts induced by the A70 treatment may not be sufficiently complete due to the higher energy concentration of the A70 diet. Further research will be conducted to determine an optimal combination of alfalfa and layer ration intermediate to the A70 and A90 regimens, for maximizing molt induction and post molt egg quality. In addition, the commercial egg industry will require more research to determine long-term effects on alfalfa and the effect on hens after longer molting periods common in industry.

CHAPTER IV

THE INFLUENCE OF A FRUCTOOLIGOSACCHARIDE (FOS) PREBIOTIC WITH ALFALFA ON *IN VITRO* FERMENTATION OF LAYING HEN CECAL BACTERIA

SYNOPSIS

Prebiotics such as fructooligosaccharide (FOS) stimulate growth and activity of colonic bacteria thus improving the host's health. The objective of this *in vitro* study was to evaluate the effects of combining a prebiotic with an alfalfa molting diet on fermentation by laying hen cecal bacteria. Cecal contents from laying hens were diluted to a 1:3000 concentration with an anaerobic dilution solution. The cecal dilution was added to serum tubes filled with ground alfalfa and layer ration with or without FOS. Samples were handled in an anaerobic hood, pressurized using a methanogen manifold and incubated at 37°C. Samples were removed at 0, 6 and 24 hours after fermentation. The trend was that fermentation increased as time increased especially when alfalfa (A) and alfalfa + FOS (AF) were evaluated in both trials. Fermentation was measured by subtracting time 0 from time 6 and time 24 and evaluating volatile fatty acid (VFA) and lactic acid (LA) concentrations. Total VFA concentrations after 6 and 24 hours showed AF treatment to have the highest concentrations and inoculum (I) and inoculum + FOS (IF) exhibited significantly ($P > 0.05$) lower total VFA concentrations than all other treatments. Similar results were shown when LA was evaluated. In trial 1, all

treatments were significantly higher than I and IF treatments whereas in trial 2, the results varied but the same trend was present with I and IF being significantly lower than all treatments except LR and AF having a greater LA concentration. These data indicate that cecal fermentation can be enhanced by the addition of FOS.

INTRODUCTION

Alfalfa is a readily available, high protein, high fiber feedstuff with one of the slowest rates of passage through the avian system (Matsushima 1972; Sibbald, 1979; Garcia *et al*. 2000). Feeding laying hens alfalfa has been suggested as an alternative to conventional molting methods (the most common being feed withdrawal). In addition to addressing animal welfare and food safety issues, alfalfa diets are desirable due to their high fermentability (Matsushima 1972).

The majority of fermentation in laying hens occurs in the ceca, which provides a stable environment for indigenous microflora such as *Bifidobacterium*, *Eubacterium*, and *Propioniabacterium* (Guo et al. 2003). The microflora ferment undigested dietary compounds such as prebiotics and plant polysaccharides to produce short chained fatty acids (SCFA) or volatile fatty acids (VFA), ammonia, carbon dioxide, methane and hydrogen (Tsukahara and Ushida 2000). SCFA such as acetate, propionate and butyrate have nutritional value to the animal as they provide energy for the hen that would otherwise not be utilized in the absence of microbial fermentation. Tsukahara and Ushida (2000) estimate 30-40% of maintenance energy for monogastrics is derived from

microbial fermentation. SCFA have been proven to control *Salmonella* in poultry and can be encouraged by the addition of prebiotics to the diet (Van Immerseel et al., 2003).

The addition of prebiotics to diets has been shown to increase fermentation both *in vitro* (Rycroft et al. 2001) and *in vivo* (Xu et al., 2003). Prebiotics were defined by Gibson and Roberfroid (1995) as some form of indigestible food ingredient that is capable of stimulating the growth of selective bacteria and results in benefits to the host. Recent concerns about antibiotic resistance have forced producers in Europe to discontinue the use of antibiotics and there is a potential for the same situation in the United States (Patterson and Burkholder, 2003; Jones and Ricke, 2003). An alternative to antibiotics for poultry production is incorporating prebiotics (Patterson and Burkholder, 2003). A commonly used prebiotic used in both in human as well as animal diets is fructooligosaccharide (FOS). FOS is a naturally occurring oligosaccharide usually of plant origin and is the only product recognized and used as a food ingredient and prebiotic (Bomba et al. 2002; Gibson and Roberfroid 1995). Due to the β-linkages possessed by FOS, it is able to resist enzymatic degradation and absorption in the upper gastrointestinal tract to reach the cecum where the majority of fermentation occurs in chickens (Gibson and Roberfroid 1995; Juskiewicz et al. 2004; Xu et al. 2003). Prebiotics have been shown to reduce pathogen colonization, alter the microbial community, prevent cancer (in mammals) and reduce cholesterol (Patterson and Burkholder, 2003).

In poultry, oligosaccharides reach the hind gut and alter lower intestinal tract physiology and function, which could be beneficial in preventing bacterial

contamination on broiler carcasses and in eggs (Orban et al., 1997). Fermentation from prebiotics include shifts in production of end products such as hydrogen, carbon dioxide, bacterial cell mass, and most importantly short-chain fatty acids (SCFA) (Cummings et al. 2001). SCFA have been shown to increase the absorption of calcium, magnesium, and iron (Gibson and Roberfroid, 1995) and to modify the bacterial ecosystem in the ceca. An i*n vitro* study by Bailey et al. (1991) showed that salmonellas were unable to metabolize FOS as a food source. In addition, FOS has been shown to serve as a fermentable substrate to promote the growth of beneficial microflora such as lactic acid bacteria and *Bifidobacterium* sp. (Juskiewicz et al., 2004; Cummings and Macfarlane, 2001; Allen et al., 1997). The objective of this study was to evaluate the effects of combininag a prebiotic (FOS) with alfalfa on fermentation of laying hen cecal bacteria.

MATERIALS AND METHODS

Diluent Preparation

Anaerobic phosphate buffer used for the cecal dilution was described by Bryant and Robinson (1961) with the addition of cysteine-HCL prior to autoclaving (Shermer et al., 1998). Ingredients were mixed, autoclaved and allowed to cool. The buffer was placed into the anaerobic chamber and allowed to reduce overnight, indicated by the loss of the pinkish color from the resazurin.

Stock Component Preparation

Two substrates, alfalfa meal (A) and layer ration (LR), were examined for fermentation properties in two trials. Alfalfa meal was obtained from a local cooperative while layer ration was obtained from the Texas A&M University Poultry Science Center feed mill in College Station, Texas (Table IV-1). Approximately 0.25 g of each substrate was added to presterilized 20 ml serum tubes. Approximately 0.02 g (7.5% FOS; Encore Technologies, Plymouth, MN) was added to FOS treated tubes (AF=alfalfa+FOS, LRF=layer ration+FOS, IF=Inoculum+FOS). A control tube with cecal inoculum only (I) was used.

Animals and Cecal Preparation

Laying hens were obtained from a commercial laying facility. Birds were housed 1 per cage at the Texas A&M University (TAMU) Poultry Science Research Center in College Station, Texas and allowed time for acclimation. During this time the birds were fed a complete layer ration (Table IV-1) *ad libitum* and allowed full access to water. All animal handling procedures were approved by the Texas A&M University Laboratory Animal Care Committee. Three hens were chosen at random and exsanguinated by CO_2 asphyxiation. The ceca of each bird were collected aseptically, placed into an empty sterile tube and immediately transported to the laboratory. The cecal contents were then squeezed into empty sterile tubes and mixed thoroughly

Table IV- 1. Composition of Texas A&M University (TAMU) layer ration and alfalfa FOS mixtures

Ingredient	TAMU layer ration [a] (LR[b])	LRF[b]	A[b]	AF[b]
	----------(g/kg) ----------			
Corn, yellow	567.18	524.64	----[e]	----[e]
Soybean meal	316.33	292.61	----[e]	----[e]
Vegetable oil	76.82	71.06	----[e]	----[e]
Mono calcium phosphate	16.86	15.60	----[e]	----[e]
Calcium carbonate	15.62	14.45	----[e]	----[e]
Methionine, 98%	1.69	1.56	----[e]	----[e]
Vitamin premix[c]	2.50	2.31	----[e]	----[e]
NaCl	2.50	2.31	----[e]	----[e]
Trace mineral premix[d]	0.50	0.46	----[e]	----[e]
Alfalfa	----[e]	----[e]	1,000.00	925.00
FOS	----[e]	75.00	----[e]	75.00
Total	1,000.00	1,000.00	1,000.00	1,000.00

[a]For diet formulation, crude fat concentrations were fixed at 100 g/kg
[b] LR = layer ration; LFR = layer ration + FOS; A= alfalfa; AF= alfalfa + FOS
[c]Provides mg/kg of diet unless otherwise noted: vitamin A, 8,818 IU; vitamin D, 2,205 IU; vitamin E, 5.86 IU; vitamin K, 2.2 IU; thiamine, 1.1 IU; riboflavin, 4.4 IU; niacin, 22 IU; pantothenic acid; choline, 500 IU; vitamin B_{12}, 0.013 IU; biotin, 0.055 IU.
[d]Trace mineral premix (Nutrius Premix Division, Bioproducts Inc., Cleveland, OH), provided as milligrams per kilogram of diet unless otherwise noted: Mn, 68.2; Zn, 55; Cu, 4.4; I, 1.1; Se, 0.1.
[e] None used

to obtain a uniform pooled sample. The pooled sample was subsequently weighed (approximately 0.1 g) into another sterile tube and placed into an anaerobic chamber (10% CO_2, 5% H_2 and 85% N_2 gas phase; Coy Laboratory Products, Ann Arbor, MI) through an airlock. Cecal contents were diluted to a 1:3000 dilution (w/vol) with anaerobic phosphate buffer. This dilution was chosen based on a preliminary study, where the 1:3000 dilution was best suitable for this study. This conclusion was based on the dilution needed to properly obtain accurate VFA and lactic acid readings.

Procedure

Tubes were prepared with substrates and FOS as described above and placed into the anaerobic hood. Cecal contents were collected and diluted with the anaerobic dilution solution and then added to the appropriate tubes while in the anaerobic state. Fermentation was simulated by placing tubes in the incubator at 37°C for 0, 6, or 24 hours. At each time point, a 2 ml aliquot was obtained for volatile fatty acid (VFA) and lactic acid (LA) concentration analysis

Statistical Analysis

Data were analyzed using a one-way analysis of variance (ANOVA) to analyze the differences among treatment groups (alfalfa, alfalfa + FOS, layer ration, layer ration + FOS, no inoculum, and inoculum only) using general linear model procedures (SAS

Institute Inc., Cary, NC, 2001) Differences among treatment groups, when significant, were compared using Duncan's multiple range test. Level of significance used in all results was $P > 0.05$.

RESULTS AND DISCUSSION

Volatile Fatty Acid and Lactic Acid Concentration

At hour 6, in both trials all treatments (A, AF, LR, LRF) had significantly ($P > 0.05$) higher acetic acid concentration (Figure IV-1) than inoculation (I) or inoculation + FOS (IF) treatments, showing that fermentation had occurred after 6 hours. At 24 hours the trend was similar; however, in both trials A and AF treatments showed the greatest increase in acetic acid concentration. The is due to more fermentative particles available after 24 hours in alfalfa when compared to layer ration, which has an average passage rate of 3 to 4 hours (Sibbald, 1979). Woodward et al. (2005) compared acetic acid concentrations from alfalfa molt diets, non molt diets and feed withdrawal molt diets and found alfalfa treatments to have overall significantly lower acetic acid concentrations than the full fed treatment. The present study used equal amounts of feed substrates and the cecal contents were consistent throughout all treatments, which is impossible when conducting an *in vivo* study.

After 6 hours of fermentation, propionic acid concentrations were significantly higher in LR and LRF than all other treatments (trial 1) and showed a similar pattern in

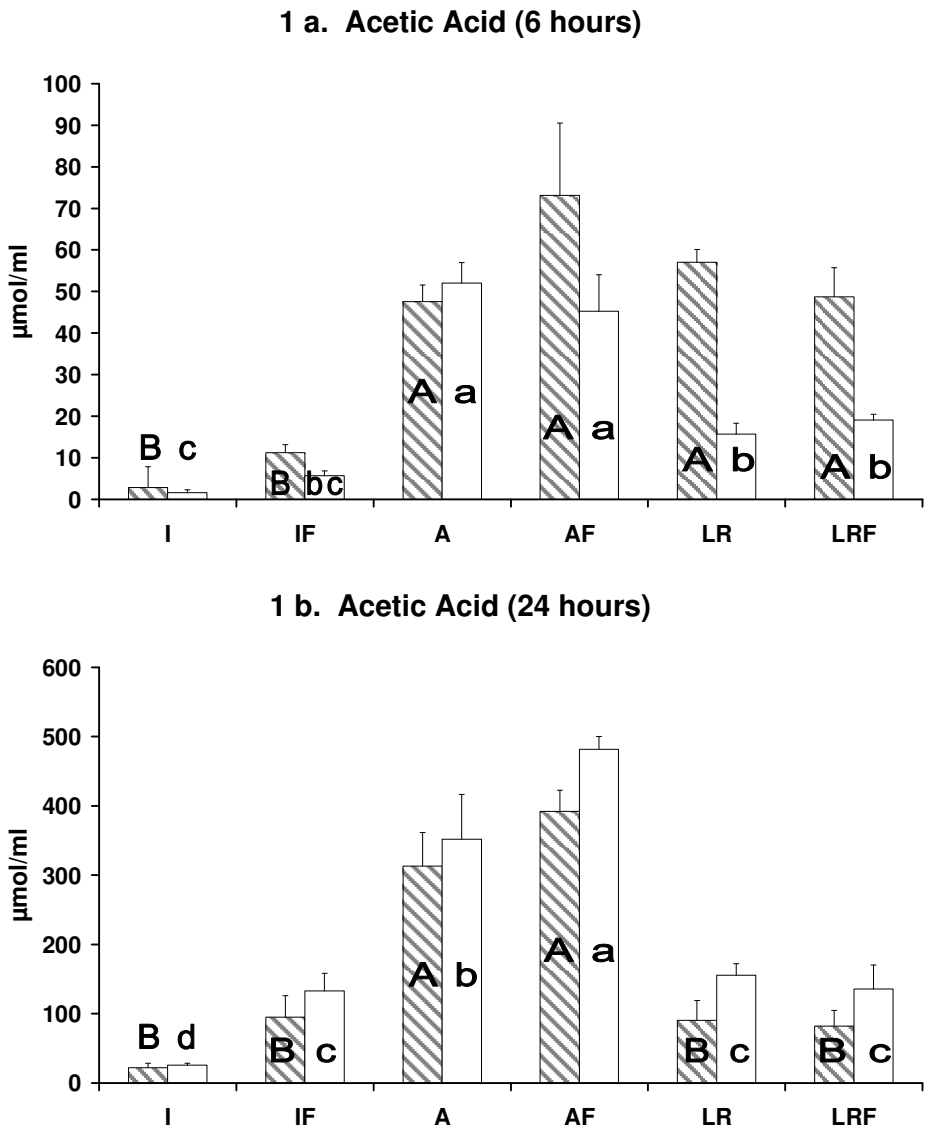

Figure IV-1. Increase in acetic acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-B] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-c] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0). Baseline values for trial 1 for respective treatments: 4.63; 5.00; 5.23; 9.10; 5.73; 3.97 µmol/mL. Baseline values for trial 2 for respective treatments: 5.03; 5.37; 8.17; 9.27; 5.27; 6.93 µmol/mL.

trial 2 with LRF having significantly higher propionic acid concentrations than all treatments except A (Figure IV-2). At 24 hours, AF had significantly higher propionic concentration than all other treatments in both trials. Propionic acid concentrations followed a pattern similar to that seen in Woodward et al. (2005) when compared to 6 hour fermentation, with non molted (full fed layer ration) hens showing significantly greater concentrations. Similar results were seen by Moore et al. (2004) where in 2 of 3 trials, propionic acid concentrations were significantly greater in FF (layer ration fed) hens when compared to feed withdrawal treated hens.

In both trials, all substrate treatments had significantly higher isobutyric acid concentrations than both I and IF treatments at 6 hours (Figure IV-3). Trial 2 exhibited a similar pattern except LRF was significantly lower than all other treatments also. After 24 hours, the general trend was similar with I and IF treatments producing significantly less isobutyric acid. While the full fed (layer ration) treated hens in Woodward et al. (2005) had higher isobutyric concentrations, they were not significantly different from the other treatments, which coincides with the results in the current trial after 6 hours fermentation.

Butyric acid concentrations were variable in trial 1 and no significant differences were seen between treatments in trial 2 after 6 hours (Figure IV-4). After 24 hours of fermentation, butyric acid concentrations increased markedly with AF being significantly higher than all other treatments in trial 1 and significantly higher than both inoculum treatments and A treatment. Butryic acid concentrations seen in this trial after

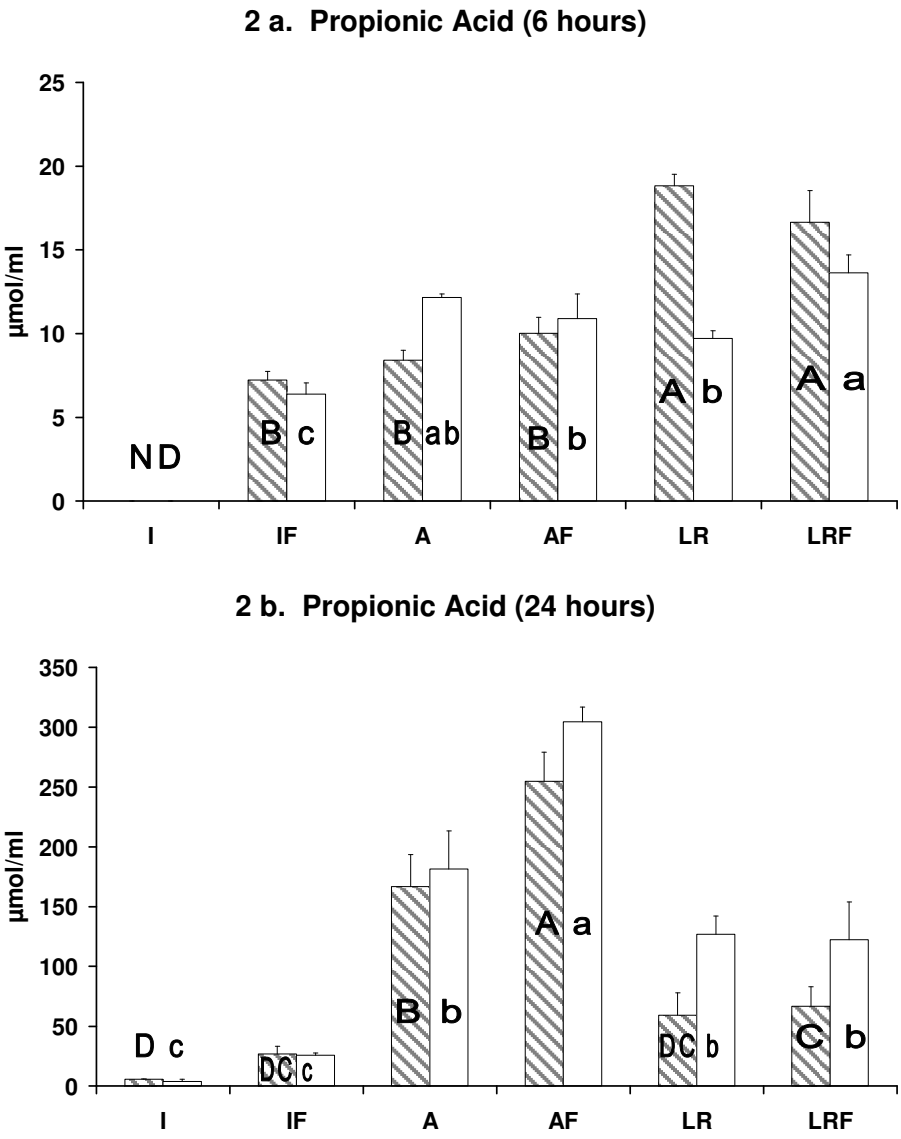

Figure IV-2. Increase in propionic acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-D] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-c] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0).
Baseline values for trial 1 for respective treatments: 0.00 µmol/mL for all treatments.
Baseline values for trial 2 for respective treatments: 0.00 µmol/mL for all treatments.

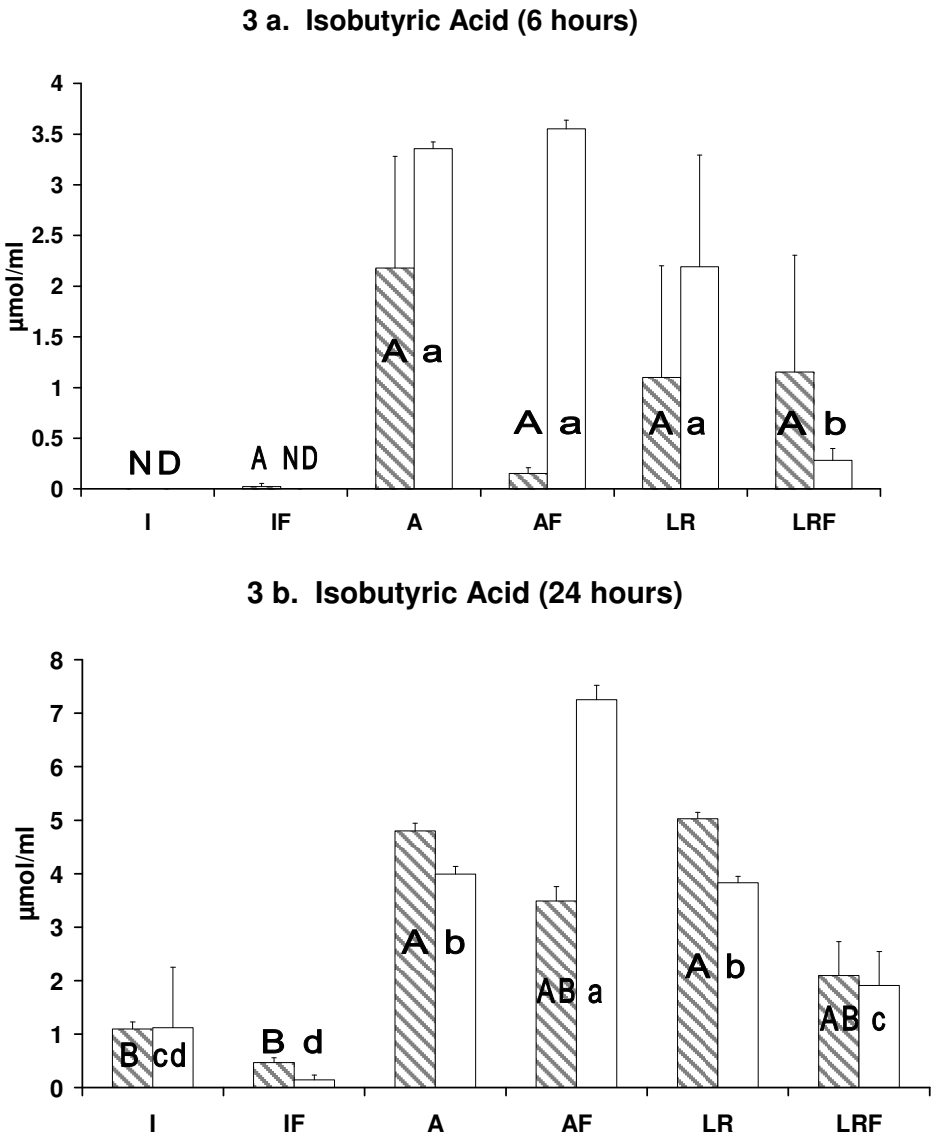

Figure IV-3. Increase in isobutyric acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-B] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-d] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0). Baseline values for trial 1 for respective treatments: 0.00; 3.37; 0.00; 3.26; 0.00; 2.23 µmol/mL. Baseline values for trial 2 for respective treatments: 0.00; 3.37; 0.00; 0.00; 0.00; 3.30 µmol/mL.

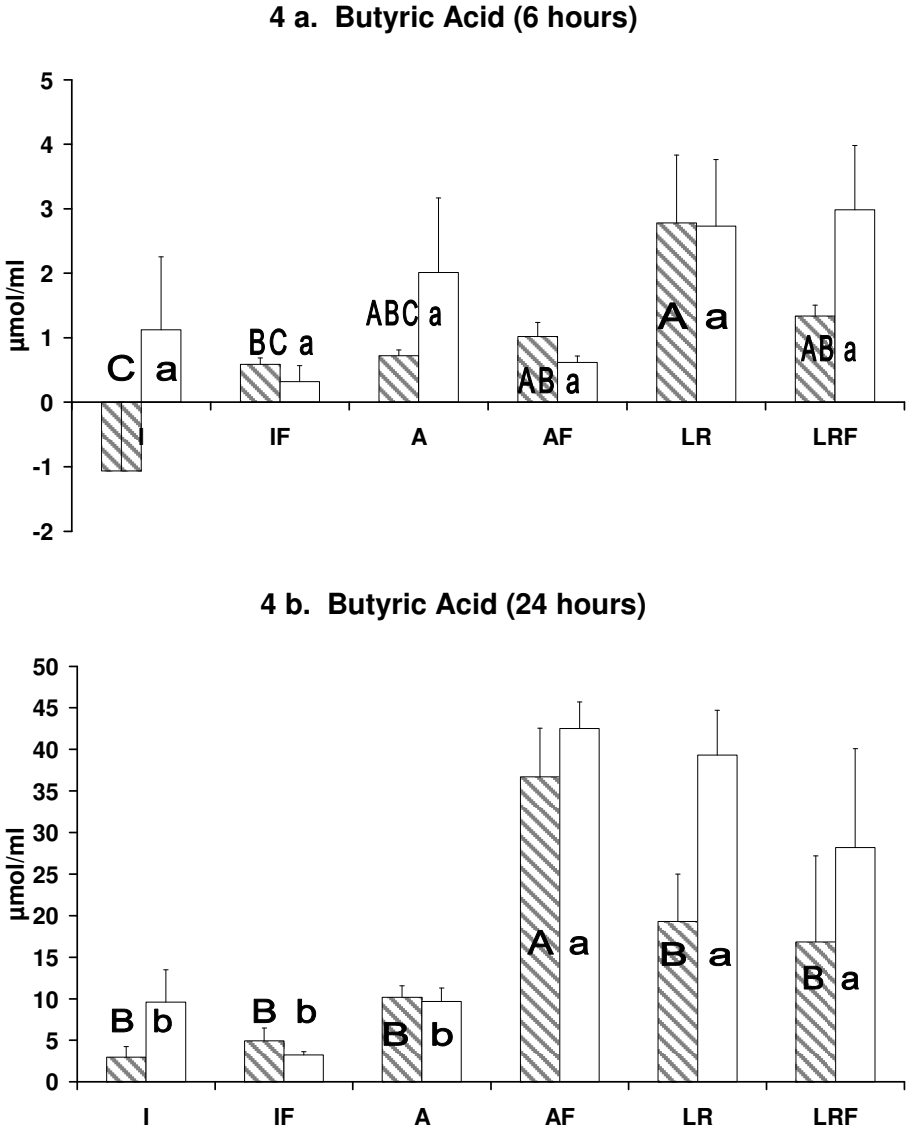

Figure IV-4. Increase in butyric acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-C] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-b] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0). Baseline values for trial 1 for respective treatments: 1.06; 3.32; 3.36; 3.33; 2.14; 3.27 µmol/mL. Baseline values for trial 2 for respective treatments: 0.00; 3.67; 2.17; 3.23; 1.10; 3.27 µmol/mL.

6 hours were similar to those concentrations seen by Moore et al. (2004) with no significant differences between treatments.

In trial 1, at 6 hours AF isovaleric acid concentrations were significantly higher than all treatments except LR and LRF (Figure IV-5). Similarly, in trial 2, AF continuation of isovaleric acid continued to be higher than the other treatments. After 24 hours of fermentation, the concentration of isovaleric acid varied between trials with no significant differences seen in trial 1 and significantly more production from AF, LR, and LRF treatments when compared to all other treatments. These results are comparable to those seen by Woodward et al. (2005) with 2 out of the 4 trials showing no significant differences between full fed (layer ration) and alfalfa treated hens.

After 6 hours of fermentation, valeric acid in I, IF, A, and LR could not be detected in the first trial and valeric acid could not be detected in all treatments except LRF in trial 2 (Figure IV-6). After 24 hours of fermentation, valeric acid could not be detected in I or IF treatments and AF showed significantly higher valeric acid concentrations. In trial 2, valeric acid concentrations were significantly higher in A, AF, LR, and LRF treatments, with no significant differences between A and IF. The results after 6 hours fermentation correspond to Woodward's (2005), findings with no detection of valeric acid in any treatment in trial 4.

When total VFA concentrations were evaluated after 6 hours of fermentation in both trials, A, AF, LR, and LRF yielded significantly greater concentrations than I and IF treatments (Figure IV-7). After 24 hours of fermentation in both trials, AF treatment had significantly higher total VFA production than all other

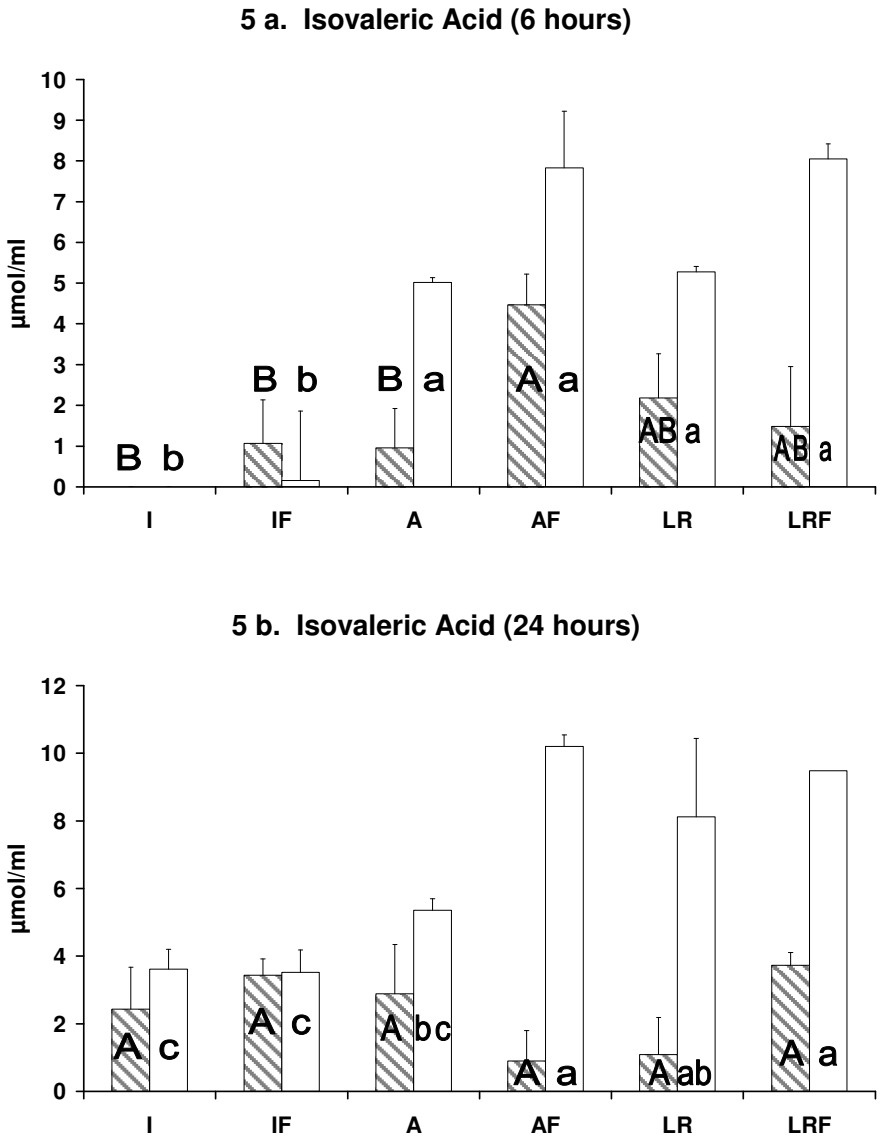

Figure IV-5. Increase in isovaleric acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-B] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-c] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0).
Baseline values for trial 1 for respective treatments: 0.00 µmol/mL for all treatments.
Baseline values for trial 2 for respective treatments: 0.00; 0.90; 0.00; 0.00; 0.00; 0.00 µmol/mL.

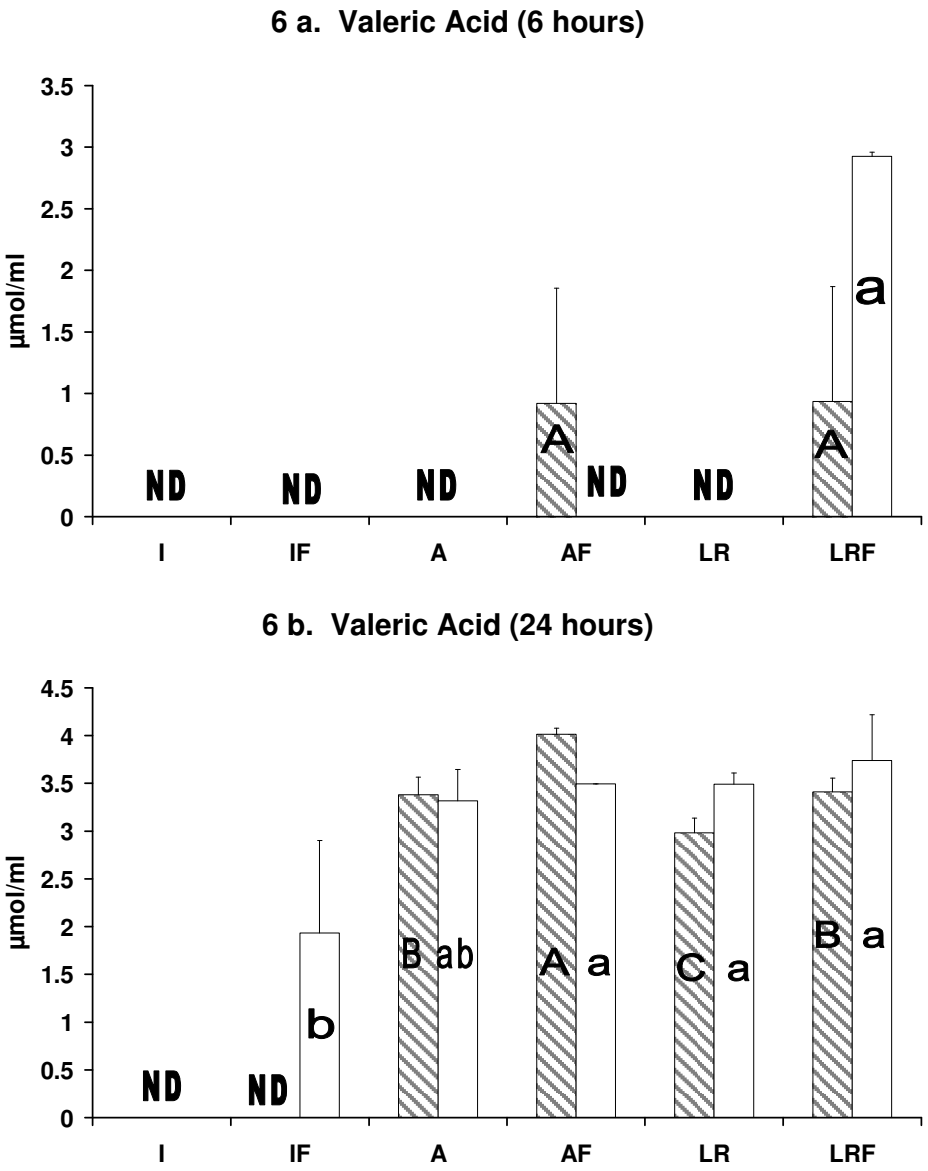

Figure IV-6. Increase in valeric acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-C] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-b] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0).
Baseline values for trial 1 for respective treatments: 0.00 µmol/mL for all treatments.
Baseline values for trial 2 for respective treatments: 0.00 µmol/mL for all treatments.

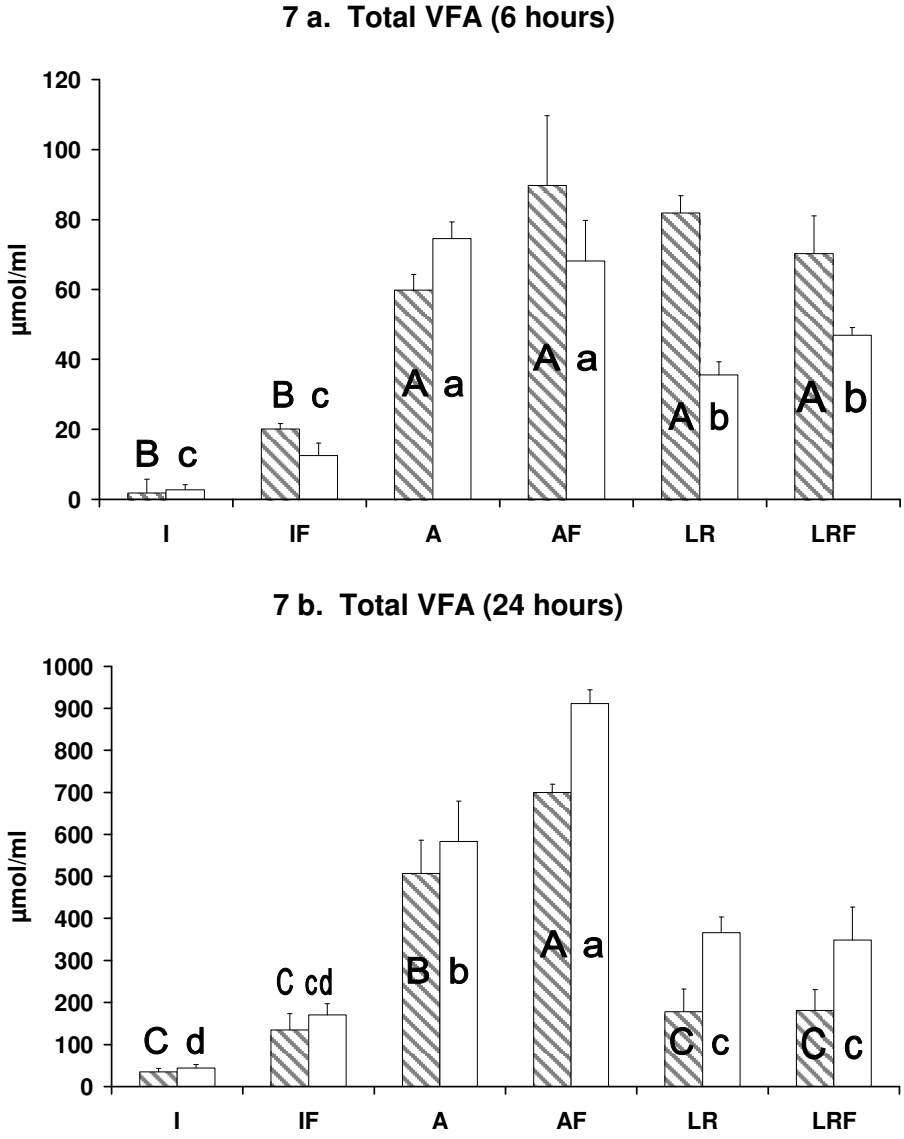

Figure IV-7. Increase in total VFA concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-C] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-d] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0). Baseline values for trial 1 for respective treatments: 5.69; 11.69; 8.59; 15.69; 7.78; 9.47 µmol/mL. Baseline values for trial 2 for respective treatments: 5.03; 13.27; 10.34; 12.5; 6.37; 12.4 µmol/mL.

treatments followed by A alone. These results show that fermentation occurred in all treatments; however, the greatest amount of fermentation occurred in the AF treatment as indicated by the overall greater total VFA production. The overall trend was the AF treatments generally having higher VFA concentrations than all other treatments and I and IF treatments having lower VFA concentrations. The results of this study did not correspond to those seen by Woodward et al. (2005) where full fed (layer ration) hens had significantly higher total VFA concentrations and alfalfa treated hens had significantly lower total VFA concentrations.

In trial 1, all treatments yielded significantly higher lactic acid concentrations than IF whereas in trial 2 both I and IF treatments showed significantly lower lactic acid concentrations than all treatments indicating fermentation occurred in all treatment groups (Figure IV-8). At 24 hours, results varied between trials; however, the trends were similar with AF exhibiting a greater lactic acid concentration than I, IF, and LR in both trials. Lactic acid concentrations were significantly greater in alfalfa treated hens in Woodward et al. (2005) as was the case in the present study with greater lactic acid concentration being shown in alfalfa treatments. Lactic acid is the primary fermentation product of lactobacillus spp. which are considered to be beneficial bacteria in the gut. Increases in lactic acid have been related to decreases in pH, thus inhibiting bacteria such as *Salmonella* from colonizing the gut (Durant et al., 1999). FOS bypasses degradation in the upper gastrointestinal tract to stimulate hind gut microflora such as *Bifidobacteria* to produce lactic acid (Tsukahara and Ushida, 2000) which has been shown to inhibit the growth of enteric pathogens (Fernandez et al., 2002).

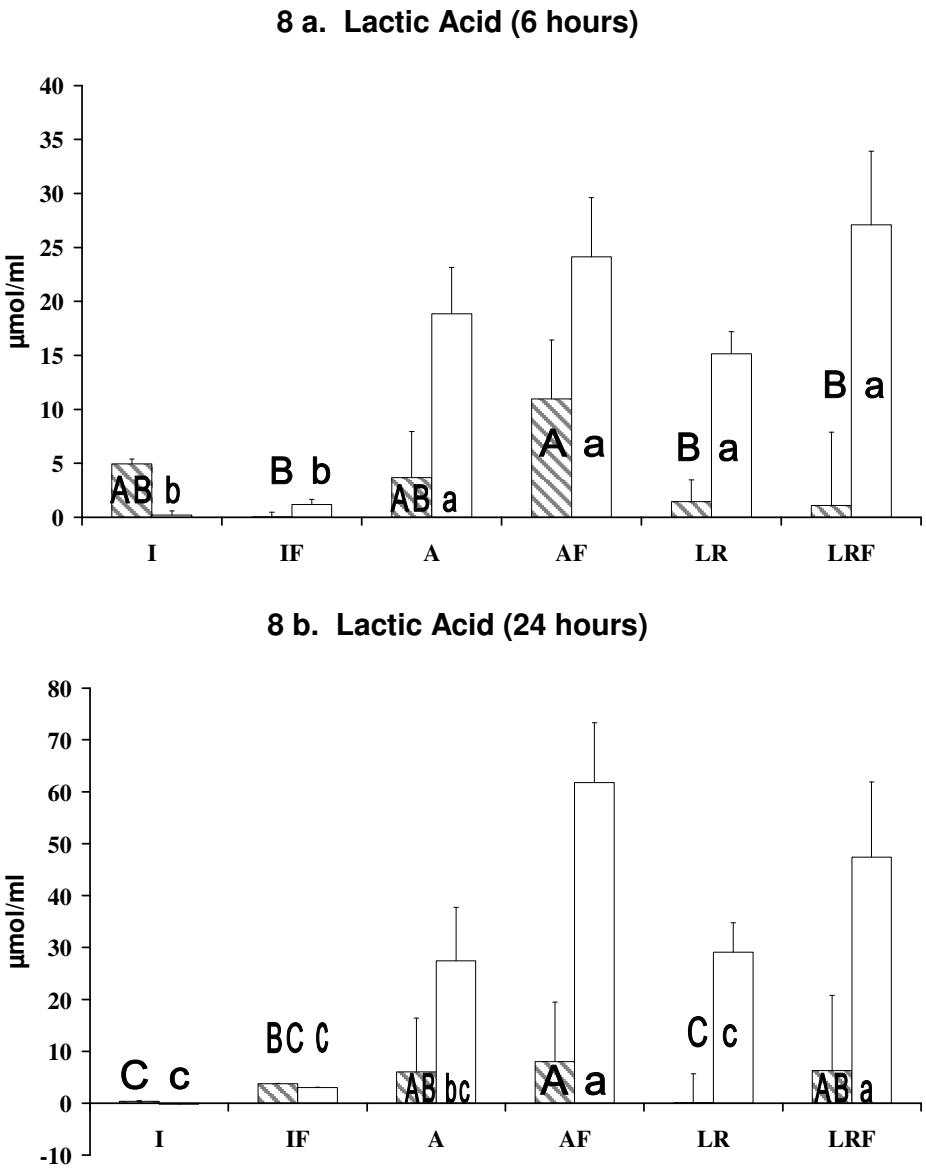

Figure IV-8. Increase in lactic acid concentration (µmol/mL) over baseline* after 6 and 24 hours fermentation.
Standard error bars are based on the average of 3 tubes per trial
[A-C] Means within trial 1 without a common letter differ significantly (P<0.05)
[a-c] Means within trial 2 without a common letter differ significantly (P<0.05)
I = inoculum only; IF = inoculum + FOS; A = alfalfa; AF = alfalfa + FOS; LR = layer ration; LRF = layer ration + FOS
*Numbers above equal concentration at 6 and 24 hours minus baseline (time 0). Baseline values for trial 1 for respective treatments: 0.00; 0.10; 2.40; 1.70; 0.47; 0.13 µmol/mL. Baseline values for trial 2 for respective treatments: 0.30; 0.70; 1.53; 2.50; 0.20; 0.37 µmol/mL.

When correlations were evaluated (Table IV-2), high correlations were seen among VFA parameters ($P < 0.0001$). The overall trend was a correlation between total VFA's and acetic acid or propionic acid. The highest correlation was seen between total VFA's and propionate with a correlation coefficient of 0.9936 and the next highest correlation was between total VFA's and acetic acid (0.9899). Butyric and lactic acids had the correlation with a coefficient of 0.6613. These results are consistent with those found in Van Der Weilen (2000). Van Der Weilen (2000) found a high presence of acetate, propionate and butyrate in the ceca of broiler chickens, thus an increase in total VFA production. The same study also showed a lack of correlation between lactobacilli numbers (indicative of lactic acid concentrations in this study) and volatile fatty acid concentrations, which was also seen in the present study.

The rationale behind the current findings is that plant protein diets such as alfalfa naturally lead to higher VFA production (Tsukahara and Ushida, 2000). In addition, alfalfa has been shown to have high fermentative properties, which also causes increased VFA production (Matsushima, 1972). The addition of FOS to the alfalfa has proven to further increase fermentation (LeBlay et al., 1999), indicative of overall higher VFA and lactic acid concentrations. Prebiotics have also been proven to be an unusable source of carbon for enterobacteria such as *Salmonella*, and *E. coli* (Bailey et al., 1991; Xu et al., 2003). While FOS can inhibit enteric pathogens, and modify the metabolic activity of the normal microflora, it does not negatively affect the indigenous bacteria. An increase in VFA concentrations has been shown to have long-term beneficial effects on host health by acting as energy sources (Guo et al., 2003) and by reducing *Salmonella*

Table IV-2. Statistical data for the correlations between volatile fatty acids (VFA) and lactic acid (LA) concentrations from laying hen cecal bacteria

	Lactic acid	Acetic acid	Propionic acid	Butyric acid
Acetic acid	0.6937****			
Propionic acid	0.7518****	0.9725****		
Butyric acid	0.6613****	0.7365****	0.8344****	
Total VFA	0.7575****	0.9899****	0.9936****	0.8064****

**** ($P < 0.0001$)

Enteritidis colonization (Woodward et al., 2005) and *Enterobacteriaceae* numbers in the ceca (Van Der Wielen, 2000). In addition, Van Der Wielen et al. (2000) suggest that VFA's such as acetate, propionate and butyrate aid in the development of the microflora in chickens.

As incubation time increased so did total VFA concentrations, with 24 hours incubation time being the peak of fermentation. Rycroft et al. (2001) also evaluated the time effects on fermentation and revealed similar results. While increases in VFA production at 6 hours were shown in the present study and at 5 hours in the Rycroft et al. (2001) study, after 24 hours of fermentation in both studies large increases in VFA's were seen. Due to the fact that the retention time of alfalfa in the gastrointestinal tract of laying hens is 24 hours (Sibbald, 1979) and with the retention time of layer ration being even shorter, it is illogical to examine the fermentative effects past 24 hours.

CONCLUSIONS

The majority of fermentation occurs in the ceca of the chicken which is home to indigenous microflora such as *Bifidobacteria*, and *Lactobacillus* (Salanitro et al., 1974). When supplied with fermentable substrates these bacteria benefit from the fermentation by-products such as VFA's. In this study, fermentation was increased when alfalfa was the feed substrate. With the addition of FOS to an alfalfa diet, fermentation increases (as indicated by increased VFA and lactic acid production). FOS is a beneficial feed additive, which not only increases fermentation to decrease pathogen colonization but

also benefits the indigenous microflora directly. In this study, FOS increased fermentation when combined with not only alfalfa but also layer ration. These results suggest that the addition of a highly fermentable feed additive such as FOS to feed substrates especially alfalfa increases *in vitro* fermentation. This study also showed that the greatest amount of fermentation occurs at 24 hours when compared to 6 hours. Fermentation after 24 hours could be evaluated, however, it is unlikely that this would be able to replicated in an *in vivo* model due to the majority of feed substrate passage rates being well under 24 hours. As indicated by the results, the addition of FOS to an alfalfa diet increased fermentation as indicated by increased total VFA's and lactic acid. Due to increases in lactic acid, the gastrointestinal ecology was altered for the benefit of the indigenous microflora. To ascertain the full benefits of this research, *in vivo* research will need to be conducted. *In vivo* studies also will help determine the optimum levels of prebiotics that should be included in a poultry diet and the complete effects FOS has on the indigenous microflora and pathogen colonization in chickens.

CHAPTER V

THE INFLUENCE OF A FRUCTOOLIGOSACCHARIDE (FOS) PREBIOTIC WITH FEED SUBSTRATES ON *IN VITRO* *SALMONELLA* TYPHIMURIUM GROWTH ON LAYING HEN CECAL BACTERIA

SYNOPSIS

The objective of this study was to investigate the effect of combining a prebiotic with feed substrates on the growth of *Salmonella* Typhimurium in an *in vitro* cecal inoculum system. Cecal contents from three laying hens were pooled and diluted to a 1:3000 concentration in an anaerobic dilution solution. The cecal dilution was added to sterile test tubes filled with alfalfa and grain with and without FOS. Two controls, cecal dilution only and anaerobic dilution solution only were used. The samples were processed in the anaerobic hood and incubated at 37°C. Samples were inoculated with *Salmonella* at 0, 6 and 24 hours after *in vitro* fermentation and then plated at 0, 6 and 24 hours after inoculation. Plates were incubated for 24 hours and enumerated. In samples inoculated at 0 hours after *in vitro* fermentation, *Salmonella* increased 64-fold from 0 to 6 hours after inoculation (beginning count 10^7 and 10^9 respectively), however, between 6 and 24 hours after inoculation, no further increase was observed. *Salmonella* counts for cecal dilution only and anaerobic dilution only at 24 hours post inoculation were

significantly lower than other treatments ($P < 0.05$). For samples inoculated at 6 hours after *in vitro* fermentation (average initial counts 10^7) *Salmonella* generally grew slowly over time (4.5-fold) with significant ($P > 0.05$) differences at 24 hours after inoculation for inoculum and no inoculum when compared to all other treatments. Samples inoculated with *Salmonella* 24 hours after fermentation showed a general decrease of *Salmonella*. At 24 hours after inoculation, grain plus FOS and alfalfa plus FOS samples (average initial counts 10^9) had significantly lower *Salmonella* counts (99.95% and 99.96% respectively). These results show 24 hour *in vitro* cecal fermentation reduced *Salmonella* growth, particularly when FOS was present.

INTRODUCTION

Non-typhoidal *Salmonella* serotypes are a significant problem for the layer industry in the United States and Europe (Holt et al., 1995; Bäumler, 2004) causing 1.4 million illnesses and 550 deaths annually in the United States (CDC, 2004). *Salmonella* colonizes the intestinal epithelium and is able to spread to a variety of organs such as the ovaries and oviducts without physical symptoms of illness being shown in the infected hens (Gast, 1994; Guard-Petter, 2001). It is important to increase fermentation especially in laying hens due to the increased susceptibility of *Salmonella* infection during the molting period and consequently an increased risk of human salmonellosis from contaminated eggs (Ricke, 2003). While the incidences of salmonellosis have decreased world wide due to improved control measures, there are new challenges facing the poultry industry including antibiotic resistance (Mead, 2004). In addition to

addressing animal welfare concerns, high fiber, low energy alfalfa molt diets (Ponte et al., 2004; Landers et al., 2005a,b) are desirable due to their high fermentation abilities by cecal microflora that are capable of limiting the *in vitro* growth of *Salmonella* Typhimurium (Matsushima, 1972; Woodward et al., 2005).

The majority of fermentation in laying hens occurs in the ceca, which provides a stable environment for indigenous microflora such as *Bifidobacterium*, *Eubacterium*, and *Propioniabacterium* (Salanitro et al. 1974; Guo et al. 2003). The microflora ferment undigested dietary compounds such as prebiotics and plant polysaccharides to produce short chained fatty acids (SCFA) or volatile fatty acids (VFA), ammonia, carbon dioxide, methane and hydrogen (Tsukahara and Ushida, 2000). SCFA have been proven to control *Salmonella* in poultry and their production can be encouraged by the addition of a prebiotic to the diet (Van Immerseel et al., 2003).

The addition of feed additives such as prebiotics has been shown to increase fermentation both *in vitro* (Rycroft et al., 2001, Donalson et al., 2004a) and *in vivo* (Xu et al., 2003). Prebiotics were defined by Gibson and Roberfroid (1995) as an indigestible food ingredient which stimulate the growth of one or a number of colonic bacteria, thus benefiting the host. To be considered a prebiotic, a food ingredient must be neither hydrolyzed nor absorbed in the upper gastrointestinal tract, be a selective substrate, be able to beneficially alter microflora, and induce luminal or systemic effects which are also beneficial to the host (Gibson and Roberfroid, 1995). A commonly used prebiotic compound used in both in human as well as animal diets is fructooligosaccharide (FOS). Fermentation of prebiotics produces end products such as

hydrogen, carbon dioxide, bacterial cell mass, and most importantly short-chain fatty acids (SCFA; Cummings et al., 2001). SCFA have been shown to increase the absorption of calcium, magnesium, and iron (Gibson and Roberfroid, 1995) and to modify the bacterial ecosystem in the ceca by lowering the pH which in turn inhibits the growth of enteric bacteria such as *Salmonella, Escherichia coli* and *Clostridium perfringens* (Cummings and Macfarlane, 2001; Patterson and Burkholder, 2003). An i*n vitro* study by Bailey et al. (1991) showed that salmonellas were unable to metabolize FOS as a nutrient source. The same study also was conducted *in vivo* and FOS was found to decrease the number of *Salmonella* positive birds as well as reducing *Salmonella* per gram of ceca (Bailey et al., 1991). In addition to inhibiting the growth of enteric bacteria, FOS has been proven to serve as a fermentable substrate to promote the growth of beneficial microflora such as lactic acid bacteria and *Bifidobacterium* sp. (Juskiewicz et al., 2004; Cummings and Macfarlane, 2001; Allen et al., 1997). Enhanced butyrate formation in the gastrointestinal tract has also been shown to alter the indigenous microflora by providing a feed substrate thus further supporting growth (Hammes and Hertel, 2002).

MATERIALS AND METHODS

Bacterial Strain

A chicken isolate of *Salmonella typhimurium* (ATCC 14028) resistant to novobiocin (NO) and nalidixic acid (NA) was used in this study. Luria-Bertani broth (LB; Difco Laboratories, Sparks, MD) was used for maintenance and growth of the strain. The bacterial strain was grown overnight in a water bath with agitation at 37°C, washed with sterile Butterfields buffer, resuspended in sterile Butterfield's buffer and diluted to an optical density of 0.300 (600 nm).

Diluent and Media Preparation

Anaerobic phosphate buffer used for the cecal dilution was described by Bryant and Robinson (1961) with the addition of cysteine-HCL prior to autoclaving (Shermer et al., 1998). Ingredients were mixed, autoclaved and allowed to cool. The buffer was placed into the anaerobic chamber and allowed to reduce overnight, indicated by the loss of the pinkish color from the resazurin. Butterfield's buffer (Difco Laboratories, Sparks, MD) prepared according to manufacturers instructions, autoclaved and used as a diluent for *Salmonella* before plating samples. Brilliant Green Agar (Difco Laboratories, Sparks, MD) with the addition of 25ug/ml of NO was prepared and autoclaved. The agar was subsequently poured into petri dishes and allowed to cool.

Stock Component Preparation

Alfalfa meal and layer ration were examined for fermentation properties against *Salmonella* Typhimurium. Alfalfa meal was obtained from a local cooperative and layer ration was obtained from the Texas A&M University Poultry Science Center feed mill (Table V-1). Approximately 0.25 g of each substrate was added to presterilized 20 ml serum tubes. Approximately 0.02 g (7.5%) FOS (Encore Technologies, Plymouth, MN) was added to FOS treated tubes and tubes were placed into the anaerobic chamber.

Animals and Cecal Preparation

Laying hens were obtained from a commercial laying facility. During this time the birds were fed a complete layer ration (Table V-1) *ad libitum* and allowed full access to water. All animal handling procedures were approved by the Texas A&M University Laboratory Animal Care Committee. Three hens were chosen at random and exsanguinated by CO_2 asphyxiation. The ceca of each bird were collected aseptically, placed into an empty sterile tube and immediately transported to the laboratory. The cecal contents were then squeezed out into empty sterile tube and mixed thoroughly to obtain a uniform pooled sample. The pooled sample was then weighed into a sterile tared tube (approximately 0.1 g) and placed into an anaerobic chamber (10% CO_2, 5% H_2 and 85% N_2 gas phase; Coy Laboratory Products, Ann Arbor, MI) through an airlock. Cecal contents were diluted to a 1:3000 dilution (w/vol) with anaerobic phosphate buffer.

Table V-1. Composition of Texas A&M University (TAMU) layer ration

Ingredient	Amount (g/kg of mash) [1]
Corn, yellow	567.18
Soybean meal	316.33
Vegetable oil	76.82
Mono calcium phosphate	16.86
Calcium carbonate	15.62
Methionine, 98%	1.69
Vitamin premix[2]	2.50
NaCl	2.50
Trace mineral premix[3]	.50
Total	1,000.00

[1]For diet formulation, crude fat concentrations were fixed at 100 g/kg
[2]Provides mg/kg of diet unless otherwise noted: vitamin A, 8,818 IU; vitamin D, 2,205 IU; vitamin E, 5.86 IU; vitamin K, 2.2 IU; thiamine, 1.1 IU; riboflavin, 4.4 IU; niacin, 22 IU; pantothenic acid; choline, 500 IU; vitamin B_{12}, 0.013 IU; biotin, 0.055 IU.
[3]Trace mineral premix (Nutrius Premix Division, Bioproducts Inc., Cleveland, OH), provided as milligrams per kilogram of diet unless otherwise noted: Mn, 68.2; Zn, 55; Cu, 4.4; I, 1.1; Se, 0.1.

Trial 1

Tubes were prepared with substrates and FOS as described above and placed into the anaerobic hood. The bacterial strain was prepared and allowed to grow overnight. Cecal contents were collected and diluted with the anaerobic dilution solution and then added to the appropriate tubes in the anaerobic hood. To evaluate the effects on fermentation *in vitro* two different fermentation times were used. The first group was inoculated with *Salmonella* Typhimurium (ST) immediately, and then samples were either collected immediately (no fermentation was allowed to occur to give a baseline level of SCFA for the study) or they were collected after 24 hours of fermentation to determine growth response. The second group was allowed 24 hours fermentation before inoculated with ST and then samples were collected immediately or allowed 24 hours further fermentation to evaluate the effects of fermentation on ST. This was done to simulate the natural environment in the gastrointestinal tract of laying hens. Fermentation occurred in the incubator at 37°C. After fermentation, the products were plated on previously prepared plates as described above and incubated for 24 hours at 37°C before counting. Treatments used in trial 1 included cecal contents combined with the following: ST (CS), alfalfa (A), alfalfa plus FOS (AF), layer ration (LR), layer ration plus FOS (LRF), and a pure culture of ST (S).

Trial 2

Preparations occurred as described in trial 1. Cecal contents were added to the appropriate tubes in the anaerobic hood. To evaluate the effects on fermentation *in vitro* two different fermentation times were used. The first group was inoculated with *Salmonella* Typhimurium (ST) immediately, and then samples were either collected immediately (no fermentation was allowed to occur to give a baseline for the study) or they were collected after 24 hours of fermentation to show determine growth response. The second group was allowed 24 hours fermentation before being inoculated with ST and then samples were collected immediately or allowed 24 hours further fermentation to evaluate the effects of fermentation on ST. This was done to simulate the natural environment in the gastrointestinal tract of laying hens. Fermentation occurred in the incubator at 37°C. After fermentation, the products were plated on previously prepared plates as described above and incubated for 24 hours at 37°C before counting. Treatments for trial 2 are shown in Table V-2.

Statistical Analysis

Data were analyzed using a one-way analysis of variance (ANOVA) to analyze the differences among treatment groups using general linear model procedures (SAS Institute Inc., Cary, NC, 2000) Differences among treatment groups, when significant, were compared using Duncan's multiple range test. Level of significance used in all results was $P < 0.05$.

RESULTS AND DISCUSSION

In vitro ST Growth

In trial 1, all substrate (alfalfa and layer ration) and FOS based treatments (ACFS, ACS, AFS, AS and CFS) showed an average of 2 Log growth while treatments without a substrate (CS, S) showed significantly decreased growth (Table V-3). A similar trend was seen in trial 2, however, significantly less growth was seen in treatments where alfalfa was combined with cecal contents indicating a synergism between the two when compared to AFS, AS and CFS treatments. CS and S treatments again showed significantly less growth than all other treatment with S having the least growth. Salmonella require substrates to sustain growth (Holt, 2003) which were not provided in the anaerobic dilution solution alone; however, cecal contents contain substrates which supported growth, while minimal, in CS treatments.

The second group of treatments was allowed to ferment for 24 hours before being inoculated with ST (Table V-4). Then samples were collected immediately after inoculation and 24 hours after inoculation. This allowed the natural microflora present in the cecal contents to ferment to evaluate the effects of fermentation on inhibition of ST growth, as by products of fermentation include short-chained fatty acids (SCFA; Tsukahara and Ushida, 2000). Inhibition of *Salmonella* growth has been seen at a pH as high as 6 (Van Immerseel et al., 2003). This is generally due to increased SCFA production which decreases the pH of the gut. Cells expend much of their energy to

Table V-2. Treatments used in trial 2 to investigate the effect of combining a prebiotic with feed substrates on the growth of *Salmonella typhimurium* in an *in vitro* model.

Treatment	Abbreviation
Salmonella pure culture	S
FOS	F
Cecal contents	C
Cecal contents + *Salmonella*	CS
Cecal contents + FOS	CF
Cecal contents + FOS + *Salmonella*	CFS
Alfalfa	A
Alfalfa + *Salmonella*	AS
Alfalfa + FOS	AF
Alfalfa + cecal contents	AC
Alfalfa + cecal contents + *Salmonella*	ACS
Alfalfa + cecal contents + FOS	ACF
Alfalfa + cecal contents + FOS + *Salmonella*	ACFS

Table V-3. Effects of *in vitro* fermentation with hen cecal contents on *Salmonella* Typhimurium growth (log counts; Trial 1).

	Treatment					
Salmonella inoculation time, Fermentation time allowed	ACFS[1]	ACS[1]	LRCS[1]	LRCFS[1]	CS[1]	S[1]
0,0	7.51±0.05[a]	7.55±0.11[a]	7.60±0.12[a]	7.60±0.11[a]	7.19±0.04[b]	7.06±0.03[b]
0,24	9.45±0.25[a]	9.40±0.24[a]	9.42±0.06[a]	9.57±0.05[a]	7.91±0.01[b]	7.84±0.07[b]
24,0	8.71±0.07[abc]	8.78±0.01[ab]	8.86±0.09[a]	8.80±0.05[a]	8.60±0.04[c]	8.64±0.04[bc]
24,24	5.40±0.20[b]	7.24±0.10[a]	6.45±0.50[ab]	5.39±0.78[b]	7.26±0.10[a]	7.28±0.04[a]

[1] ACFS=Alfalfa+cecal contents+FOS+Salmonella; ACS=Alfalfa+cecal contents+Salmonella; LRCS = Layer ration+cecal contents+Salmonella; LRCFS= Layer ration+cecal contents+FOS+Salmonella; CS+Cecal contents+Salmonella; S=Salmonella only

[a-c] Means with same letter in a row are not significantly different

Table V-4. Effects of *in vitro* fermentation with hen cecal contents on *Salmonella* Typhimurium growth after being inoculated at times 0 and 24. (log counts; Trial 2).

	Treatment						
Salmonella inoculation time, Fermentation time allowed	ACFS[1]	ACS[1]	AFS[1]	AS[1]	CFS[1]	CS[1]	S[1]
0,0	7.30±0.07[a]	7.41±0.10[a]	7.41±0.13[a]	7.14±0.11[a]	7.42±0.04[a]	7.08±0.28[a]	6.96±0.13[a]
0,24	6.88±0.21[b]	7.14±0.07[b]	8.46±0.12[a]	8.26±0.05[a]	8.22±0.17[a]	6.44±0.18[c]	0.00±0.00[d]
24,0	2.57±1.49[b]	4.82±0.20[ab]	5.46±0.24[a]	6.15±0.93[a]	2.15±1.24[b]	4.64±0.20[ab]	4.61±0.21[ab]
24,24	0.00±0.00[a]	2.33±1.37[a]	3.60±2.08[a]	3.81±1.28[a]	0.00±0.00[a]	3.61±1.24[a]	2.15±1.25[a]

[1] ACFS=Alfalfa+cecal contents+FOS+Salmonella; ACS=Alfalfa+cecal contents+Salmonella; AFS=Alfalfa+FOS+Salmonella; CFS=Cecal contents+FOS+Salmonella; CS+Cecal contents+Salmonella; S=Salmonella only

[a-d] Means with same letter in a row are not significantly different

compensate for the decreased pH and are not able to use energy for necessary metabolic processes (Van Immerseel et al., 2003). ST growth in trial 1 was inhibited by all treatments with the most inhibition seen in both alfalfa and layer ration combined with FOS. The effects of fermentation on ST inhibition were more immediate in trial 2 than in trial 1, with the most dramatic decreases seen in ACFS and CFS treatments after an additional 24 hours of fermentation for a total of 48 hours of fermentation. However, the decreases seen in these two treatments were not significantly different from any other treatments.

CONCLUSIONS

The results of this study provide evidence that the addition of FOS to alfalfa and layer rations may inhibit *Salmonella* Typhimurium growth after fermentation has been allowed to occur. The addition of FOS to substrate diets in combination with cecal contents act in a synergistic manner to increase SCFA production, thus decreasing *Salmonella* growth. The microenvironment created by cecal bacteria after fermentation is highly unfavorable to pathogens such as *Salmonella* due to a decreased pH (Van Immerseel et al., 2003). In addition, FOS is unable to be used as a source of carbon for enteric bacteria such as *Salmonella* (Bailey et al., 1991).

CHAPTER VI

THE INFLUENCE OF A FRUCTOOLIGOSACCHARIDE (FOS) PREBIOTIC COMBINED WITH ALFALFA MOLT DIETS ON THE GASTROINTESTINAL TRACT OF LAYING HENS AND *SALMONELLA* ENTERITIDIS

SYNOPSIS

Molting is a natural process, which birds undergo to rejuvenate their reproductive organs. The United States Poultry industry commonly uses feed withdrawal to effectively induce molt however, recent animal welfare concerns have encouraged producers to seek alternative molting methods due to the increased susceptibility of *Salmonella* Enteritidis (SE). SE infections affect nearly 1.4 million people in the United States annually with over 500 deaths and annually cost consumers nearly 2.3 billion dollars. The primary mode of infection includes contaminated shell eggs and other poultry products. Contaminated eggs stem from colonization in the gastrointestinal tract and during stressful conditions such as feed withdrawal. Some alternative methods to feed withdrawal include feeding high fiber diets such as alfalfa. Alfalfa is a high protein, low energy feedstuff that is well balanced in amino acids and rich in vitamins and is a comparable alternative to the commonly used molting practice of feed withdrawal. Previous studies have shown that alfalfa is effective at molt induction and provides equivalent post molt production numbers and quality when compared to feed

withdrawal. Alfalfa has also been shown to decrease the incidence of *Salmonella* Enteritidis (SE) colonization, however, the SE levels detected in some birds suggest inconsistency in the promotion of gut microflora antagonistic to SE. The objective of this study was to evaluate the effects of combining a prebiotic with an alfalfa molting diet on the gastrointestinal tract of laying hens and *Salmonella* Enteritidis colonization.

INTRODUCTION

Salmonellosis is a foodborne disease that affects over 1.4 million people each year in the United States alone, of which more than 500 are fatal (CDC, 2004). Frenzen et al. (1999) estimate the annual cost of foodborne salmonella infection is nearly 2.3 billion dollars. The majority of this is due to loss of productivity in the workforce and medical bills (Frenzen et al., 1999). The two serotypes that cause the majority of the cases are *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST). SE cases are generally believed to be derived from shell eggs from chickens. These eggs come from hens that appear perfectly healthy but carry the disease in their gastrointestinal and reproductive tracts, which is then transmitted to the interior of the egg prior to shell formation; in addition, these contaminated eggs are indistinguishable from non-contaminated, normal eggs. This fact along with undercooking contaminated eggs leads to SE infection. Management practices, such as feed withdrawal molting methods, increase the susceptibility of SE infection in the hen, (Poppe, 1999) as indicated by increased intestinal shedding and dissemination of SE to organs such as the ovary, liver, spleen, and crop.

Due to the increased popularity of molting practices, alternative diets need to be developed to help resist SE and maintain a healthy microbial ecosystem during a forced molt. Providing a hen with a low energy diet such as alfalfa has been shown to effectively induce molt and helps maintain *Lactobacillus* population in the crop and ceca. With this research, we combined alfalfa molt diets with FOS and examined the effects of SE colonization on internal organs and the effect on pH, VFA and lactic acid production. This research is significant, because it provides the poultry industry better molting strategies to reduce *Salmonella Enteritidis* (during molting), effectively rejuvenates the hen, alleviates the stresses associated with feed withdrawal molting methods and enables the poultry industry to maintain its economic advantage. The commercial egg industry commonly uses induced molt procedures to rejuvenate flocks for a second or third laying cycle and to increase profits. According to Bell (2003), approximately 75% of commercial laying facilities in the United States used an induced molt program to rejuvenate flocks for increased productivity. Implementing an induced molt program can result in a 30% higher profit margin for producers, when compared to an all-pullet operation (Bell, 2003). In addition to increased profit margins, an induced molt rejuvenates the hen's reproductive tract to produce higher quality eggs, which are more marketable (Keshavarz and Quimby, 2002). The main purpose of molting is to cease egg production in order for the hens to enter a non-reproductive state which increases egg production and egg quality post molt (Webster, 2003).

While there are several molting methods, feed withdrawal has been the most popular due to ease of application, economic benefits, and agreeable post-molt

performance (Keshavarz and Quimby, 2002; Bell, 2003). Feed withdrawal (FW) molting methods are seen as logical because wild birds exhibit similar behavior when they undergo a natural molt; they lose as much as 40% of their body weight while refusing food until the later stages of the molt (Mrosovsky and Sherry, 1980). However, recent concerns have been raised about animal welfare during the feed withdrawal period because it is thought to be harmful to the hens (Webster, 2003) and increases the incidence of salmonellae (Hinton et al., 2000). Historically, researchers have examined alternative diets to FW that provide similar benefits while not altering the health and behavior of the animals or increasing susceptibility of salmonellae as feed withdrawal increases the incidence of SE (Holt, 1993). General dietary modification strategies have involved either constructing diets that are deficient in some nutrients such as sodium or contain an excess of a particular compound such as zinc or feeding alternative feedstuffs such as wheat middlings (Seo et al., 2001), and alfalfa (Woodward et al., 2005; Landers et al., 2005a,b).

Alfalfa is a readily available, high protein, high fiber feedstuff with one of the slowest rates of passage through the avian system (Matsushima, 1972; Sibbald, 1979; Garcia et al., 2000). Alfalfa has proven to be an effective alternative molting diet as it induces molt and produces comparable post molt egg production and qualities when compared to FW (Donalson et al., 2005). In addition to addressing animal welfare and food safety issues as an alternative molt diet, alfalfa diets are desirable due to their high fermentation properties by cecal microflora that are capable of limiting the *in vitro*

growth of *Salmonella* Typhimurium when alfalfa is present (Donalson et al., 2004a,b; Matsushima, 1972).

The gastrointestinal tract of poultry has been studied at a great extent (Van Der Wielen et al., 2000, Salanitro et al., 1974, Apajalahti et al., 2004) and has proven to be a remarkable physiological structure. The gastrointestinal tract includes the structures of the digestive tract, which are responsible for nutrient and water absorption, fermentation and waste excretion. Within these structures is a diverse, complex microbial ecosystem with the majority of bacteria residing in the cecum (Salanitro et al., 1974). The cecum in birds is much different when compared to mammals, due to the increased surface area, which is helpful in hydrolysis, absorption, and fermentation (Vispo and Karasov, 1997). Most of the bacteria in the cecum are considered strict anaerobes and include species such as *Lactobacilli, Bifidobacterium* and *Propioniabacterium* (Salanitro et al., 1974). The microflora in the ceca work together to maintain a stable ecosystem in order to form a natural resistance to infections produced by enteric pathogens (Hentges, 1983). This is accomplished by forming a physical barrier to keep intestinal bacteria in check and protect against enteric pathogens by discriminating between enteric and resident microflora (Lu and Walker, 2001). Enteric pathogens possess specialized processes, which allow them to penetrate the intestinal epithelium. Inside the intestinal epithelium the pathogen can adhere to the surface, colonize and establish permanent residence, which can cause disease if not prevented by the natural microflora (Lu and Walker, 2001).

Cecal microflora ferment undigested dietary compounds such as prebiotics and plant polysaccharides to produce short chained fatty acids (SCFA) or volatile fatty acids (VFA), ammonia, carbon dioxide, methane and hydrogen (Tsukahara and Ushida, 2000). SCFA such as acetate, propionate and butyrate have nutritional value to the animal as they provide energy for the hen that would otherwise not be utilized in the absence of microbial fermentation. Tsukahara and Ushida (2000) estimate 30-40% of maintenance energy for monogastrics is derived from microbial fermentation. The production of SCFA has been proven to control *Salmonella* and other enteric pathogens in poultry and can be enhanced by the addition of prebiotics to the diet (Donalson, 2004b).

Prebiotics were first defined by Gibson and Roberfroid (1995) as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." The addition of prebiotics to diets has been shown to increase fermentation both *in vitro* (Rycroft et al., 2001, Donalson et al., 2004a,b) and *in vivo* (Xu et al., 2003). A commonly used prebiotic compound used in both in human as well as animal diets is fructooligosaccharide (FOS) which is a naturally occurring oligosaccharide usually of plant origin and is the only product recognized and used aa a food ingredient and prebiotic (Bomba et al., 2002; Gibson and Roberfroid, 1995). Due to the β-linkages possessed by FOS, it is able to resist enzymatic degradation and absorption in the upper gastrointestinal tract to reach the cecum where the majority of fermentation occurs in chickens (Gibson and Roberfroid, 1995; Juskiewicz et al., 2004; Xu et al., 2003). Fermentation of prebiotics produces end products such as hydrogen,

carbon dioxide, bacterial cell mass, and most importantly short-chain fatty acids (SCFA) (Cummings et al., 2001). SCFA have been shown to increase the absorption of calcium, magnesium, and iron (Gibson and Roberfroid, 1995) and to modify the bacterial ecosystem in the ceca by lowering the pH, which in turn can inhibit the growth of enteric bacteria such as *Salmonella, Escherichia coli* and *Clostridium perfringens* (Cummings and Macfarlane, 2001). An i*n vitro* study by Bailey et al. (1991) showed that salmonellas were unable to metabolize FOS as a food source. In addition to inhibiting the growth of enteric bacteria, FOS has been proven to serve as a fermentable substrate to promote the growth of beneficial microflora such as lactic acid bacteria and *Bifidobacterium* sp. (Juskiewicz et al., 2004; Cummings and Macfarlane, 2001; Allen et al., 1997.). The objective of this study was to evaluate the effects of combining a prebiotic with an alfalfa molting diet on the gastrointestinal tract of laying hens and *Salmonella* Enteritidis colonization.

MATERIALS AND METHODS

Molting Procedure

A total of 60 laying hens were obtained from a commercial laying facility. Cloacal swab samples were collected from each hen and examined for salmonellae by successive culturing in tetrathionate (TT) broth (Difco Laboratories, Detroit, MI) and brilliant green agar (BGA) plates as described by Andrews et al. (1992). *Salmonella* spp- positive hens were eliminated from the study. Laying hens were placed in wire

layer cages (1 hen per cage) and provided free access to water and a balanced, unmedicated, corn-soybean mash layer ration (TAMU layer ration) that met or exceeded NRC requirements (1994). This diet was formulated to provide 2,818 kcal of ME/kg, 16.5% CP, 3.5% calcium, and 0.48% available phosphorus. Before use, 3 randomly selected 25-g samples of the feed was cultured successively in buffered peoptone water, tetrathionate broth, and BGA as described by Andrews et al. (1992) and examined for salmonellae. The hens were allowed to acclimate for a minimum of 1 week followed by complete random allocation to five treatment groups of 12 hens each, designated as follows: (1) feed withdrawal (molted, FW); (2) non molted control (full fed, FF); (3) 90% alfalfa / 10% TAMU layer ration (A90); (4) 90% alfalfa / 10% TAMU layer ration plus 0.375% FOS (L); or (5) 90% alfalfa / 10% TAMU layer ration plus 0.75% FOS (H). The hens were then housed in approved facilities at the USDA-ARS, College Station, Texas, under a protocol approved by the USDA-ARS Animal Care and Use Committee.

On day 4 of each study, all hens in each treatment group were challenged by crop gavage with 1 mL of inoculum containing approximately 10^5 colony-forming units (CFU) of NONA (Novobiocin/Nalidixic)-resistant SE. The challenge dosage approximates the 5.6×10^4 cfu dose reported to be the mean infectious dosage for SE in nonmolted hens (Holt, 1993). On day 9 of the study, 6 hens from each treatment group were euthanitized and the crop, ceca, liver, spleen, and ovary aseptically excised. The crop, ceca, liver, spleen and ovary of each hen were then cultured for SE. Following the molting period, the remaining 6 hens from each treatment group were placed on a maintenance diet and observed for intestinal shedding of SE.

Crop Lactic Acid Concentrations and pH

Crop lactic acid concentration and pH were determined as described by Durant et al. (1999). Crop pH was determined by insertion of a sterile glass pH electrode through an incision in the crop wall ensuring the electrode remained in contact with the crop mucosal surface. Each crop was aseptically excised, cut open, and blended with 10 mL of sterile Butterfield's Buffer for 1 minute in a Stomacher 80 blender. Samples of blended crop were analyzed for lactic acid concentrations (Moore et al., 2004).

Cecal Volatile Fatty Acid and Lactic Acid Concentrations

The concentrations of volatile fatty acids (VFA; acetic, propionic, butyric, isobutryic, valeric and isovaleric acids) in the cecal contents were determined by gas-liquid chromatography as described by Corrier et al. (1990). The analysis was conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Model 110 Gas Chromatograph, SR1 Instruments, Torrence, CA). Each sample peak profile was integrated and quantified relative to an internal standard of methylbutyric acid placed in the same sample. Lactic acid concentrations were determined by an enzymatic method (Hohorst, 1974).

Crop Colonization by SE

One milliliter of blended crop sample was transferred into 10 mL Rappaport-Vassiliadis broth (RV; EM Science, Gibbstown, NJ) and incubated for 24 hours at 42°C. After incubation, the broth was streaked onto NONA-BGA plates, incubated for an additional 24 hours at 37°C and examined for the presence of suspect SE colonies. Suspect colonies were identified as SE serologically using *Salmonella* O antiserum group D, factors 1,9,12.

Cecal Colonization by SE

One cecum from each hen was cut into several pieces, placed in 30 mL of RV broth, shaken vigorously, and incubated for 24 hours at 42°C. After incubation, the broth will be streaked on NONA-BGA plates, incubated for an additional 24 hours at 37°C, and examined for the presence of suspect SE colonies. Suspect colonies will be identified as SE serologically using *Salmonella* O antiserum group D, factors 1,9,12.

Liver, Spleen, and Ovary Colonization by SE

Liver, spleen, and ovary specimens were minced with scissors and cultured. The organ samples were incubated for 24 hours at 42°C in RV broth. After incubation, the broth was streaked onto NONA-BGA plates, incubated for an additional 24 hours at

37°C and examined for the presence of suspect SE colonies. Suspect colonies were identified as SE serologically using *Salmonella* O antiserum group D, factors 1,9,12.

SE Colony-Forming Units Per Gram of Crop and Cecal Contents

The contents of the crop and one cecum from each hen were serially diluted and spread plated on NONA-BGA plates at dilutions 10^1 through 10^8. The plates were incubated for 24 hours at 37°C, after which the number of cfu of SE per gram of crop or cecal content were determined and SE colonies were confirmed by using *Salmonella* O antiserum group D, factors 1,9,12.

Intestinal Shedding of SE

Hens were assayed for intestinal shedding of SE on days 4, 10, 17, and 24 post challenge for six hens per treatment group. The birds were sampled using a modification of a procedure described by Seo *et al.* (2001). Aluminum foil sheets were placed under each hen for approximately one hour and the secretions were collected. Approximately 0.5 ml of the sample was weighed and added to a dilution tube containing 4.5 ml sterile Butterfield's Buffer (Difco Laboratories, Sparks, MD). The aliquot was then serially diluted at 10^1 through 10^8 dilutions and plated on NONA-BGA plates. The plates were incubated for 24 hours at 37°C, after which the number of cfu of SE per gram of intestinal shedding was determined and SE colonies were confirmed by using

Salmonella O antiserum group D, factors 1,9,12. The remaining sample was added to 25 ml RV broth for selective enrichment and incubated for 24 hours at 42°C, at which time it was plated on NONA-BGA plates and incubated at 37°C for another 24 hours. The plates were then examined for the presence of suspect SE colonies. Suspect colonies were identified as SE serologically using *Salmonella* O antiserum group D, factors 1,9,12.

Statistical Analysis

Differences among treatment groups, when significant, were compared using Duncan's multiple range test. Differences in the VFA, and lactic acid concentrations, and crop, cecal, liver, spleen and ovary colonization by SE were evaluated by analysis of variance using the general linear models procedures. Significant differences were further separated using Duncan's multiple range test and commercial statistical analysis software (SAS, 2001). All data was analyzed by individual trial and statistical analyses considered significant at ($P < 0.05$).

RESULTS AND DISCUSSION

Laying Hen Response to Treatments

Feed intake (Table VI-1) was significantly ($P > 0.05$) greater in full fed (FF) non-molted hens in trials 1 and 2 (84.13, 93.69 g/hen daily, respectively). There were no

significant differences between any molting treatments in either trial, however, the overall feed intake in molt treated hens was significantly higher in trial 2. When hens undergo a natural molt as described by Mrosovsky and Sherry (1980) they voluntarily undergo anorexia, thus greatly decreasing or even ceasing feed intake. The decrease in feed intake of alfalfa molt diets can also be attributed to reduced palatability or change in energy levels as alfalfa is a low-energy diet (Biggs et al., 2004; NRC, 1994). With a decreased feed intake, body weights also decrease markedly (Table VI-1). In trial 1, FF non-molted hens had significantly lower body weight loss (48.50g) when compared to all molted treatments, which were not significantly different from each other. Trial 2 also showed significantly less body weight loss (-14.18g) in FF treated hens. However, while FW hens still showed significantly greater body weight loss than FF and no significant differences from A90 and L treatments, it showed significantly greater body weight loss than H treated hens in trial 2. Body weight is closely associated with a complete rejuvenation of the reproductive tract (Baker et al., 1983). Hens with 25-30% body weight loss during a molt have been shown to have increased post molt performance (Baker et al., 1983). Body weight loss is directly related to the ovarian weight loss as half the body weight is due to the regression of the ovaries and oviduct (Sherry et al., 1980). Ovary regression (Table VI-1) is an important factor which influences both post molt egg production and egg quality (Biggs et al., 2004). In both trials, FF treated hens had significantly greater ovary weights typical of non-molted hens while all molted hens showed no significant differences between treatments. These

Table VI-1. Effects of nonmolting and molting with and without alfalfa and FOS on feed intake, body weight loss and ovary weight of hens.

		Treatment				
	Item	A90[1]	FF[1]	FW[1]	H[1]	L[1]
Trial 1						
	Feed intake (g/hen daily)	11.39±3.06[bc]	84.13±3.57[a]	NA[3]	12.63±1.00[b]	11.32±4.99[bc]
	Body weight loss (g)	317.00±31.20[a]	48.50±51.74[b]	417.83±39.28[a]	380.00±21.49[a]	356.00±109.83[a]
	Ovarian weight[2]	0.69±0.90[b]	3.13±0.27[a]	0.86±0.17[b]	0.61±0.11[b]	0.65±0.11[b]
Trial 2	Feed intake (g/hen daily)	15.11±6.05[bc]	93.69±14.97[a]	NA[3]	21.06±2.96[b]	15.72±0.79[bc]
	Body weight loss (g)	374.75±23.89[ab]	-14.18±42.23 [c]	441.92±19.31[a]	346.08±18.84[b]	430.25±34.97[ab]
	Ovarian weight[2]	0.44±0.12[b]	2.73±0.69[a]	0.63±0.17[b]	0.49±0.11[b]	0.85±0.23[b]

[a-c] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).
[1]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.
[2]As a percentage of body weight (ovary weight / body weight) x 100.
[3]NA = non applicable.

results are comparable to those seen in studies by Woodward et al. (2005) and Landers et al. (2005).

Crop pH and Lactic Acid

Crop pH and lactic acid concentrations were measured in hens following the 9 d molt and are shown in Table VI-2. The primary bacteria in the crop are lactobacillus spp. which produce lactic acid as their main product. No significant differences in lactic acid concentrations were seen in either trial. When measuring crop pH in trial 1, FF treated hens had significantly lower pH's when compared to all other treatments which were not significantly different from each other. Similarly there were no significant differences in any treatments in trial 2. Durant et al. (1999) showed that a decreased feed intake reduces lactobacillus populations thus decreasing crop pH as seen in this study.

SE Colonization of the Crop

Percent SE colonization in the crop results varied greatly between trials (Table VI-3). Trial 1 showed A90 and L treated hens having significantly greater SE colonization % than all other treatments, whereas trial 2 showed FF treated hens to have a significantly lower % SE colonization in the crop than FW treated hens. H, L, and A90 treatments did not differ significantly from wither FW or FF in trial 2. When cfu/g

Table VI-2. Effects of nonmolting and molting with and without alfalfa and FOS on crop pH and lactic acid concnetrations

Item		Treatment				
		A90[1]	FF[1]	FW[1]	H[1]	L[1]
Trial 1						
	Crop pH	5.69±0.21[a]	4.77±0.12[b]	6.05±0.29[a]	5.60±0.20[a]	6.12±0.37[a]
	Lactic acid (mmol/mL)	33.73±10.94[a]	43.08±12.96[a]	37.42±17.43[a]	11.98±1.67[a]	12.12±2.97[a]
Trial 2	Feed					
	Crop pH	5.31±0.11[a]	4.76±5.59[a]	5.59±0.26[a]	5.53±0.10[a]	5.68±0.08[a]
	Lactic acid (mmol/mL)	21.30±5.45[a]	22.65±4.27[a]	13.48±1.80[a]	26.38±11.0[a]	31.47±10.24[a]

[a-c] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6)

[1]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

Table VI-3. Effects of nonmolting and molting with and without alfalfa and FOS on *Salmonella entericia* serovar Enteritidis (SE) crop colonization of hens

Item	Treatment[1]				
	A90[2]	FF[2]	FW[2]	H[2]	L[2]
Trial 1					
Positive hens per total	1/6 (17%)[a]	0/6 (0%)[b]	0/6 (0%)[b]	0/6 (0%)[b]	1/6 (17%)[a]
Log_{10} cfu / g	0.74±0.57[bc]	0.00±0.00[c]	0.95±0.00[a]	0.65±0.49[b]	0.61±0.45[bc]
Trial 2					
Positive hens per total	2/6 (33%)[ab]	0/6 (0%)[b]	4/6 (83%)[a]	1/6 (17%)[ab]	2/6 (33%)[ab]
Log_{10} cfu / g	1.07±0.71[ab]	0.00±0.00[b]	1.96±0.51[a]	0.68±0.68[ab]	1.03±0.71[ab]

[a-c] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).
[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.
[2]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

counts were examined in trial 1, FW hens had significantly greater counts (0.95) while FF hens had significantly lower counts (0.00) than all other treatments. In trial 2, the significance between groups was similar to that of the % SE colonization with FW having significantly higher counts (1.96) and FF showing significantly lower counts (0.00) and all other molted treatments (A90, H, L) not showing significant differences from any treatments. The crop serves as a major habitat for SE colonization, which has been shown to increase during feed withdrawal (Hargis et al., 1995).

SE Colonization of the Ceca

Table VI-4 shows SE colonization of the ceca. Trial 1 results showed significantly greater % colonization in FW treated hens when compared to all other treatments which were not significantly different from each other. Trial 2 showed similar results with FW treated hens having significantly higher % SE colonization than FF but not with other molted treatments. H and L treatments while not being significantly different from FW hens were also not significantly different from FF treated hens in trial 2. Trial 1 showed no significant differences when Log_{10} cfu/g were examined. Trial 2 exhibited similar cfu/g as % SE colonization with FW having significantly higher (3.16) cfu/g counts than FF and H treatments but cfu/g counts did not differ significantly from L and A90 treatments. In general, FF hens had significantly less SE colony forming units/g, whereas, FW hens had significantly greater cfu/g than

Table VI-4. Effects of nonmolting and molting with and without alfalfa and FOS on *Salmonella entericia* serovar Enteritidis (SE) cecal colonization of hens

Item	Treatment[1]				
	A90[2]	FF[2]	FW[2]	H[2]	L[2]
Trial 1					
Positive hens per total	1/6 (17%)[b]	0/6 (0%)[b]	6/6 (100%)[a]	2/6 (33%)[b]	2/6 (33%)[b]
Log_{10} cfu / g	0.74±0.57[a]	0.00±0.00[a]	0.95±0.00[a]	0.65±0.49[a]	0.61±0.45[a]
Trial 2					
Positive hens per total	5/6 (83%)[a]	0/6 (0%)[b]	5/6 (83%)[a]	3/6 (50%)[ab]	3/6 (50%)[ab]
Log_{10} cfu / g	2.05±1.06[ab]	0.00±0.00[b]	3.16±1.57[a]	0.38±0.23[b]	1.08±0.87[ab]

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).
[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.
[2]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

other treatments and all other molted treatments fell between the two. Similar results were seen in Woodward et al (2005) and Moore et al. (2004).

SE in the Liver, Spleen and Ovaries

Compared with FF (liver: 0%, spleen:0%, ovaries: 0%) the number of SE positive hens significantly increased in FW treatment trial 1: liver: 100%; spleen: 67%, ovaries: 100% and in trial 2: liver: 67%, spleen: 67%, ovaries: 50% (Table VI-5). When A90, H and L treatments were compared to FF in trial 1, no significant differences were seen in the organs. The number of SE positive cultures in the livers of molt treatment hens were all higher than FF treatment in trial 2. There were no significant differences between FF or FW when compared to all other treatments (A90, H, L) in SE positive cultures of the spleen. In trial 2, the number of SE positive cultures in the ovaries from H treatment (83%) were significantly greater than FF treated hens, but not significantly different from any other treatments (A90, FW, L). A90, L, and FW treatments were not significantly different from FF treated hens when SE positive cultures in the ovaries were examined. The increase in organ invasion of FW hens is due to the absence of feed in the gastrointestinal tract which results in a decrease of peristalsis muscle contractions and mucin production (Sturkie, 1965). The lack of feed in the gastrointestinal tract is also directly related to the increase in SE colonization (Holt and Porter, 1992). Seo et al. (2001) reported that by providing some form of bulk in the gastrointestinal tract, hens can clear an infection more readily than if the gut were empty. By providing alfalfa in

Table VI-5. Effects of nonmolting and molting with and without alfalfa and FOS on *Salmonella entericia* serovar Enteritidis (SE) colonization of the liver, spleen and ovary of hens

Item	Treatment[1]				
	A90[2]	FF[2]	FW[2]	H[2]	L[2]
Trial 1					
Liver	0/6 (0%)[b]	0/6 (0%)[b]	6/6 (100%)[a]	2/6 (33%)[b]	2/6 (33%)[b]
Spleen	1/6 (17%)[ab]	0/6 (0%)[b]	4/6 (67%)[a]	1/6 (17%)[ab]	2/6 (33%)[ab]
Ovary	2/6 (33%)[b]	0/6 (0%)[b]	6/6 (100%)[a]	2/6 (33%)[b]	2/6 (33%)[b]
Trial 2					
Liver	5/6 (83%)[a]	0/6 (0%)[b]	6/6 (100%)[a]	5/6 (83%)[a]	5/6 (83%)[a]
Spleen	3/6 (50%)[ab]	0/6 (0%)[b]	4/6 (67%)[a]	3/6 (50%)[ab]	1/6 (17%)[ab]
Ovary	3/6 (50%)[ab]	0/6 (0%)[b]	3/6 (50%)[ab]	5/6 (83%)[a]	3/6 (50%)[ab]

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).

[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.

[2]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

the diet, the SE was unable to fully colonize and was cleansed from the tract. However, in trial 2, some organs did become colonized at greater rates than seen in trial 1. This can be explained by a decreased feed intake when compared to FF hens. Seo et al. (2001) explained that partial feed withdrawal by hens themselves can result in partial effects of diets (such as alfalfa), on SE infection.

Cecal VFA Profile of Controls and Alfalfa and FOS Diets

Concentrations of acetic acid (Figure VI-1a) were ($P < 0.05$) significantly lower in the ceca of FW treated hens in trial 1 when compared to all other molt treatments. However, in trial 2, the results were quite different with FF hens exhibiting significantly greater ($P < 0.05$) acetic acid concentrations in the ceca than all other treatments.

There were no ($P < 0.05$) significant differences in propionic acid concentrations (Figure VI-1b) in the ceca of alfalfa molted hens in either trial. In trial 1, propionic acid concentrations were significantly ($P < 0.05$) lower than all alfalfa molt diets. In trial 2, only FF treated hens exhibited significantly greater propionic acid concentrations than FW hens.

Concentrations of isobutyric acid (Figure VI-1c) were significantly higher in H and L treated hens when compared to FW hens in trial 1. No significant differences in isobutyric concentrations were seen between any treatments in trial 2.

Butyric acid concentrations were significantly lower in FW hens when compared to all other treatments in trial 1 (Figure VI-1d). H treated hens showed significantly

greater butyric acid concentration in the ceca when compared to FW and FF treated hens. In trial 2, FF hens exhibited significantly greater butyric acid concentration than FW hens while all alfalfa molt diets were not significantly different when compared to FF and FW treated hens.

No significant differences between any treatments were seen when 2-methylbutyric acid concentrations were measured in either trial (Figure VI-1e). Similarly, there were no significant differences in isovaleric acid concentrations between any treatments in trial 1. When isovaleric acid concentrations were measured in trial 2, FF and FW treated hens showed significantly greater concentrations than A90 treated hens while H and L treatments did not differ significantly from any treatment (Figure VI-2a).

Concentrations of valeric acid were significantly lower in the ceca of FW treated hens than in the ceca of all alfalfa molt treated hens in trial 1 (Figure VI-2b). However, in trial 2, valeric acid concentrations in the ceca of FF hens were significantly greater than valeric acid concentrations in the ceca of FW hens. Alfalfa molted hens did not exhibit significant differences in valeric acid concentration when compared to either FW or FF treated hens.

Total VFA concentrations were significantly lower in the ceca of FW hens than in all alfalfa molt treatments, but not FF treated hens in trial 1 (Figure VI-2c). Trial 2

yielded quite the opposite results when total VFA concentrations were examined. The total VFA concentrations in the ceca of FF hens were significantly greater than all molt treatments. The results from trial 1 are similar to the results seen by Moore et al. (2004) where no significant differences were found in total VFA's in molted and non molted treated hens whereas the results of trial 2 correlated with the results of Woodward et al (2005) where FF hens exhibited significantly greater total VFA concentrations than hens molted by alfalfa and FW hens.

Concentrations of lactic acid were significantly greater in all 3 alfalfa molt diets than FF or FW hens in trial 1 (Figure VI-2d). Trial 2 yielded similar results except only FW treated hens had significantly lower lactic acid concentrations than all 3 alfalfa molt diets. This trend is consistent with the findings of Woodward et al. (2005) which found increased lactic acid concentration in alfalfa molt diets when compared to FF or FW treatments.

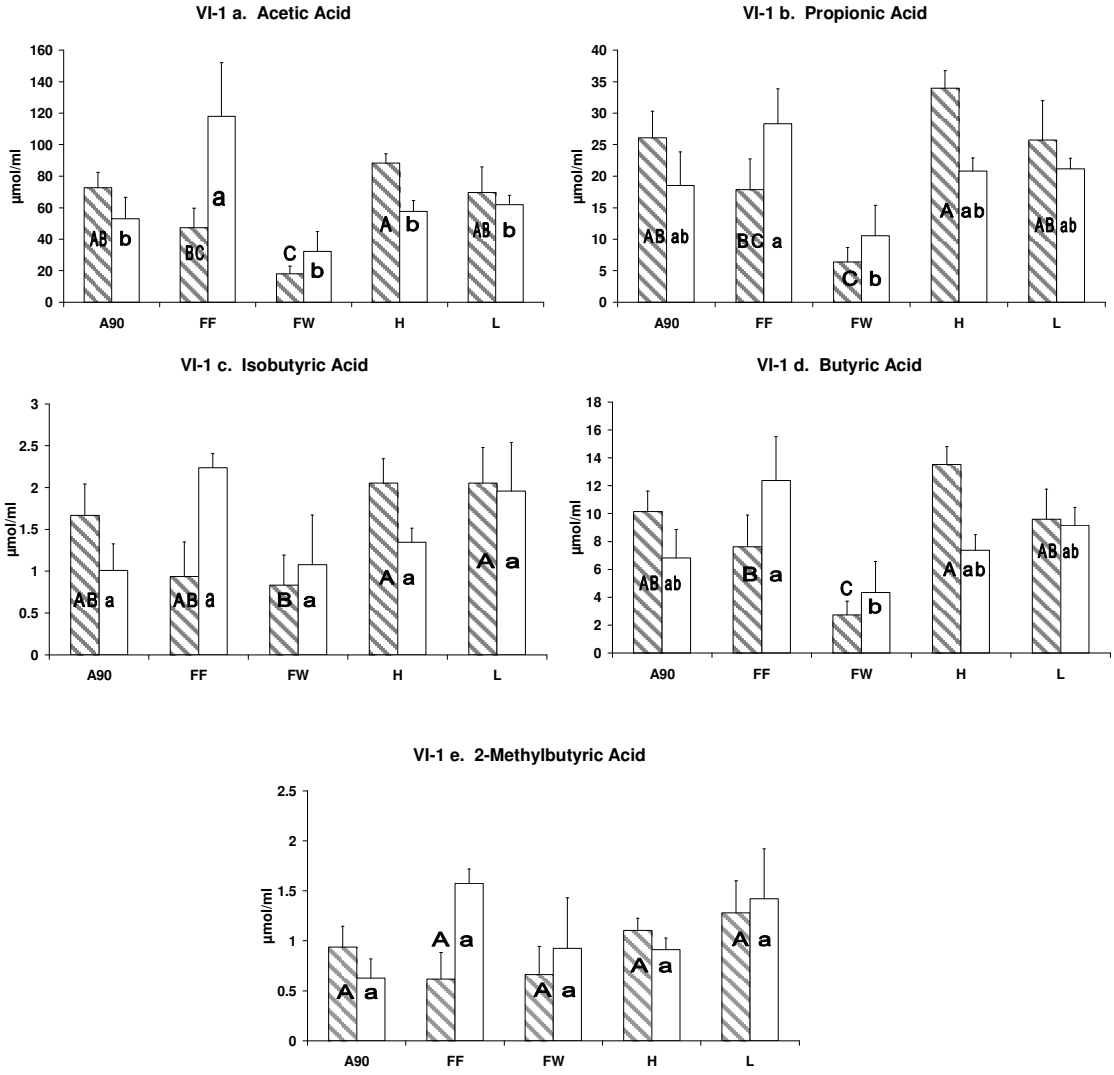

Figure VI-1. Effects of nonmolting and molting diets with and without alfalfa and FOS on cecal volatile fatty acids (VFA; µmol/mL). [A-C]Means within trial 1 without a common letter differ significantly ($P < 0.05$); [a-b]Means within trial 2 without a common letter differ significantly ($P < 0.05$). A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

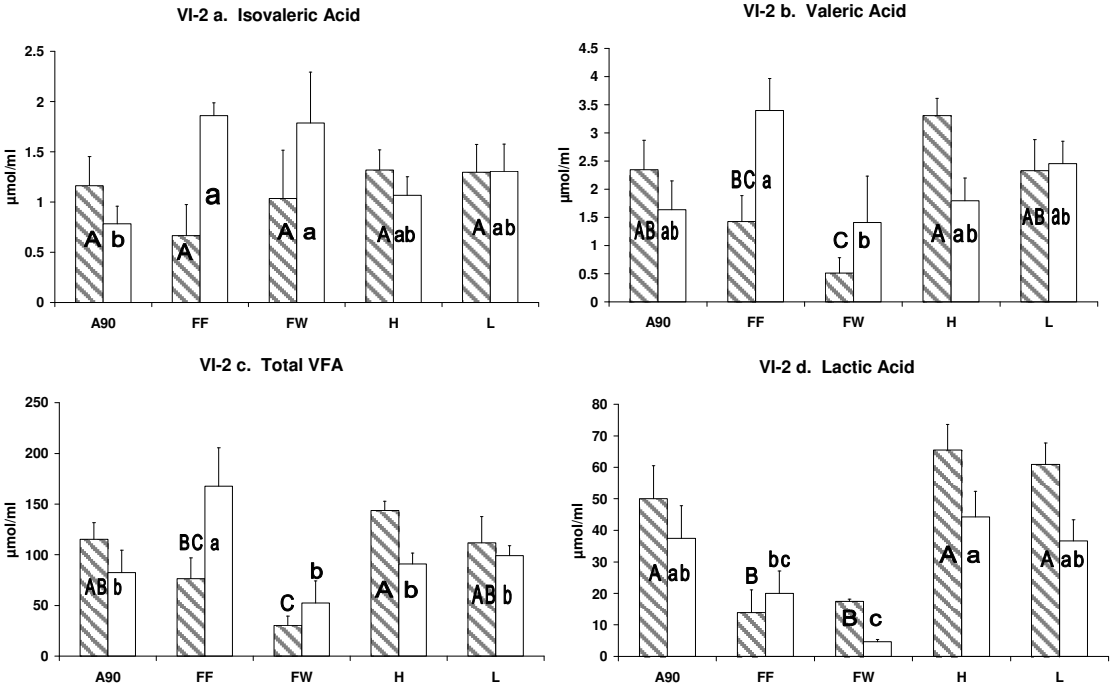

Figure VI-2. Effects of nonmolting and molting diets with and without alfalfa and FOS on cecal volatile fatty acids (VFA) and lactic acid concentrations (µmol/mL). $^{A-C}$Means within trial 1 without a common letter differ significantly ($P < 0.05$); $^{a-b}$Means within trial 2 without a common letter differ significantly ($P < 0.05$). A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

Intestinal Shedding of SE

The log SE numbers and % positive cultures from intestinal shedding is presented in Table VI-6 (trial 1) and Table VI-7 (trial 2). In trial 1, there were no significant differences in % positive cultures on any days (8, 14, 21, or 28). The only significant differences seen in trial 1 were seen on d 8 when FW hens shed significantly more SE than FF or L treated hens. There were no significant differences seen on any other days in log counts. The trend, however followed a similar pattern as seen in SE colonization of the organs with more organisms shed by FW hens than FF and all other molted treatments falling between the two. Trial 2 yielded similar results with no significant differences in % organisms shed on days 8, 21, or 28. However, on d 14, A90 hens shed significantly more SE (60%) than all other treatments which all shed 0%. Similar results were seen on d 21, however, the results were not significantly different. On d 28, the SE positive cultures and log counts increased in A90, H, and L treated hens when compared to the 2 previous sampling dates. The authors conclude that while the conditions were static and aseptic techniques were used, the birds were reinfected as *Salmonella* is airborne and can be easily transmitted from bird to bird, especially when housed in confined spaces. The overall trend of FW hens shedding more SE than FF hens is consistent with results from Seo et al. (2001).

Table VI-6. Effects of nonmolting and molting with and without alfalfa and FOS on *Salmonella entericia* serovar Enteritidis (SE) intestinal shedding (Trial 1)

Item	Treatment[1]				
	A90[2]	FF[2]	FW[2]	H[2]	L[2]
Day 8	5/6 (83%)[a] 2.03±0.60[ab]	3/6 (50%)[a] 0.48±0.21[b]	5/6 (83%)[a] 3.17±1.27[a]	4/6 (67%)[a] 1.49±0.77[ab]	3/6 (50%)[a] 0.77±0.44[b]
Day 14	0/6 (0%)[a] 0.00±0.00[a]	0/6 (0%)[a] 1.12±1.12[a]	1/6 (17%)[a] 0.16±0.16[a]	1/5 (20%)[a] 0.19±0.19[a]	1/6 (17%)[a] 0.16±0.16[a]
Day 21	1/6 (17%)[a] 1.34±0.83[a]	0/6 (0%)[a] 0.67±0.67[a]	1/6 (17%)[a] 0.16±0.16[a]	1/6 (17%)[a] 0.33±0.33[a]	0/6 (0%)[a] 0.00±0.00[a]
Day 28	1/6 (17%)[a] 1.61±0.97[a]	0/6 (0%)[a] 2.78±.098[a]	1/6 (17%)[a] 0.96±0.61[a]	1/6 (17%)[a] 1.14±1.14[a]	2/6 (33%)[a] 2.37±1.21[a]

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).

[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.

[2]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

Table VI-7. Effects of nonmolting and molting with and without alfalfa and FOS on *Salmonella entericia* serovar Enteritidis (SE) intestinal shedding (Trial 2)

	Treatment[1]				
Item	A90[2]	FF[2]	FW[2]	H[2]	L[2]
Day 8	1/4 (25%)[a] 1.13±1.13[b]	2/5 (40%)[a] 0.38±0.23[b]	4/5 (80%)[a] 3.90±1.42[a]	1/6 (17%)[a] 0.16±0.16[b]	3/6 (50%)[a] 0.78±0.44[b]
Day 14	3/5 (60%)[a] 1.18±0.38[a]	0/6 (0%)[b] 0.00±0.00[b]	0/6 (07%)[b] 0.00±0.00[b]	0/6 (0%)[b] 0.00±0.00[b]	0/6 (0%)[b] 0.00±0.00[b]
Day 21	1/4 (25%)[a] 0.24±0.24[a]	0/6 (0%)[a] 0.00±0.00[a]	0/6 (0%)[a] 0.00±0.00[a]	0/6 (0%)[a] 0.00±0.00[a]	0/6 (0%)[a] 0.00±0.00[a]
Day 28	0/6 (0%)[a] 1.74±1.01[a]	0/6 (0%)[a] 0.00±0.00[b]	0/6 (0%)[a] 0.00±0.00[b]	2/5 (40%)[a] 0.59±0.40[ab]	1/5 (20%)[a] 0.40±0.40[ab]

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$; n=6).
[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.
[2]A90 = 90% alfalfa / 10% layer ration; FF = full fed, non molted; FW = feed withdrawal; H = 90% alfalfa / 10% layer ration + 0.75% FOS; L = 90% alfalfa / 10% layer ration + 0.375% FOS.

CONCLUSIONS

Salmonellosis affects over 1.4 million people each year with one major cause being shell eggs from laying hens. Molting by withdrawing feed has been shown to increase the occurrence of *Salmonella* Enteritidis; consequently, alternative molting diets are being sought. One such alternative is the inclusion of alfalfa into a molt diet. Alfalfa is readily available, high in protein and fiber and has been proven to limit SE colonization *in vivo* (Woodward et al, 2005) and *in vitro* (Donalson et al, 2004). The addition of FOS to molt diets has proven to further limit SE colonization (Donalson et al, 2004) and promote the growth of beneficial microflora such as lactic acid bacteria (Allen et al., 1997; Cummings and Macfarlane, 2001). This study further supports the data presented by Woodward et al (2005) showing that alfalfa molt diets achieved comparable ovary reduction to feed withdrawal hens, as well as providing a gut fill which aided in the limitation of SE colonization in the ceca as well as the liver, spleen and ovaries. The addition of FOS to this diet further increased total VFA and lactic acid concentrations, although not significantly higher than A90. These results could have been affected by feed intake by alfalfa treated hens. Feed intake in alfalfa treated hens may have been affected by saponins which are undesirable compounds found in alfalfa that have been shown to decrease feed intake (Matshishma, 1972). The addition of layer ration in the A90 diets has been shown to increase feed intake (Donalson et al, 2005); however, the correlation between amount of layer ration included in a diet and SE colonization has not been examined. As there were few significant differences between

hens fed 0.75% FOS (H) and 0.375% FOS (L) it may be possible to feed a lower amount of FOS yet still retain the benefits of a higher dosage. If feed intake were increased, perhaps lower levels of FOS could be fed with the same benefits.

CHAPTER VII

CONCLUSIONS

Salmonellosis is a foodborne disease that affects over 1.4 million people each year in the United States alone, of which more than 500 are fatal (CDC, 2004). Frenzen et al. (1999) estimate the annual cost of foodborne salmonella infection is nearly 2.3 billion dollars in the United States. Molting has been shown to increase the susceptibility of laying hens to SE thus increasing the risk of human salmonellosis. Alternative molt diets are presently being developed to alleviate these food safety concerns as well as animal welfare concerns, as the primary method of molting is feed withdrawal.

The use of alfalfa as a viable molt diet has proven to be acceptable according to the experiments presented here and by other researchers. Alfalfa combined with layer ration at different ratios has proven to induce molt, increase postmolt egg quality and postmolt egg production as well as the conventional feed withdrawal method. This is greatly beneficial to the egg industry, as alfalfa is readily available and inexpensive. Alfalfa has also proven to be highly fermentable, especially in the presence of FOS, as seen in the first *in vitro* study. The second *in vitro* study continued experimentation with alfalfa combined with FOS and found the combination to greatly decrease the incidence of *Salmonella*. An *in vivo* study was conducted to bring all the previous studies together. In this study, hens responded positively to the alfalfa molt diet as seen in the previous *in*

vivo study. In addition, alfalfa diets were shown to decrease organ colonization of *Salmonella* Enteritidis, which was further decreased by the presence of FOS.

These data show that alfalfa is a practical alternative molt diet which not only induces molt and shows comparable post molt performance, but also reduces the colonization of *Salmonella* by increasing fermentation in the gastrointestinal tract, especially the ceca.

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APPENDIX

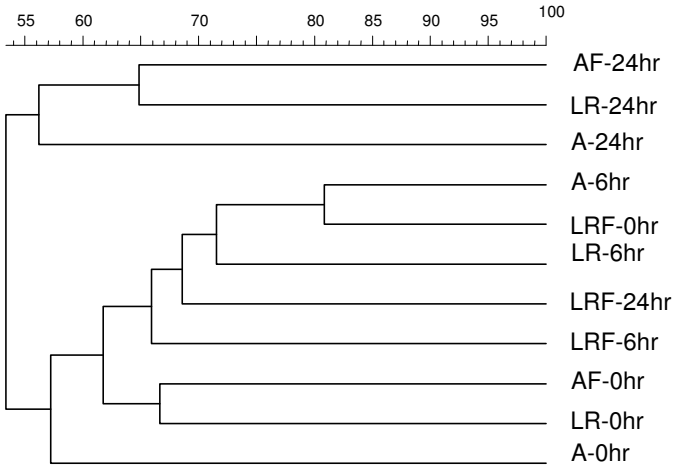

Figure A-1. Denaturing gradient gel electrophoresis gel of dietary treatments (AF = alfalfa + FOS; LR = layer ration; A = alfalfa; LRF = layer ration + FOS) at 3 time points. Relatively similarity of band patterns is indicated by their grouping o the dendrogram and the percentage coefficient (bar).

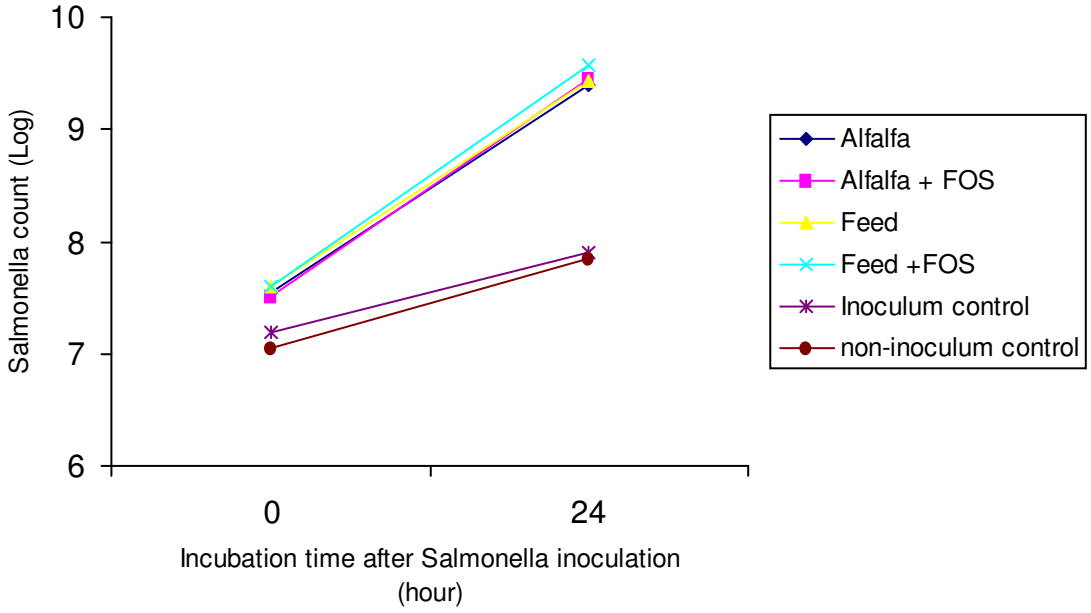

Figure A-2. Effects of *in vitro* fermentation with hen cecal contents on *Salmonella* Typhimurium growth when *Salmonella* was inoculated at 0 hr *in vitro* fermentation (Trial 1).

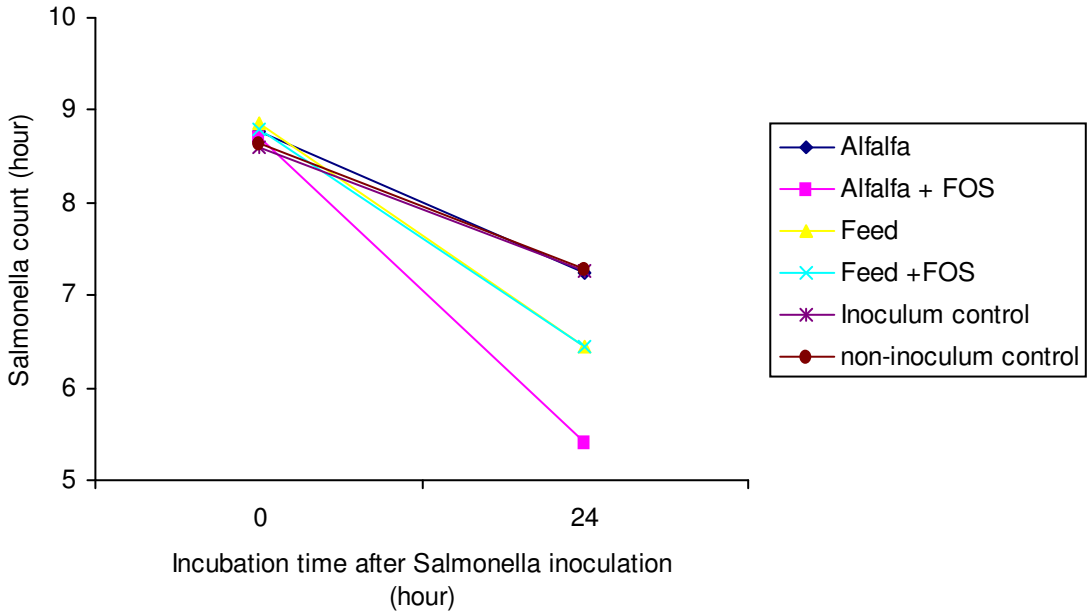

Figure A-3. Effects of *in vitro* fermentation with hen cecal contents on *Salmonella* Typhimurium growth when *Salmonella* was inoculated at 24 hr *in vitro* fermentation (Trial 1).

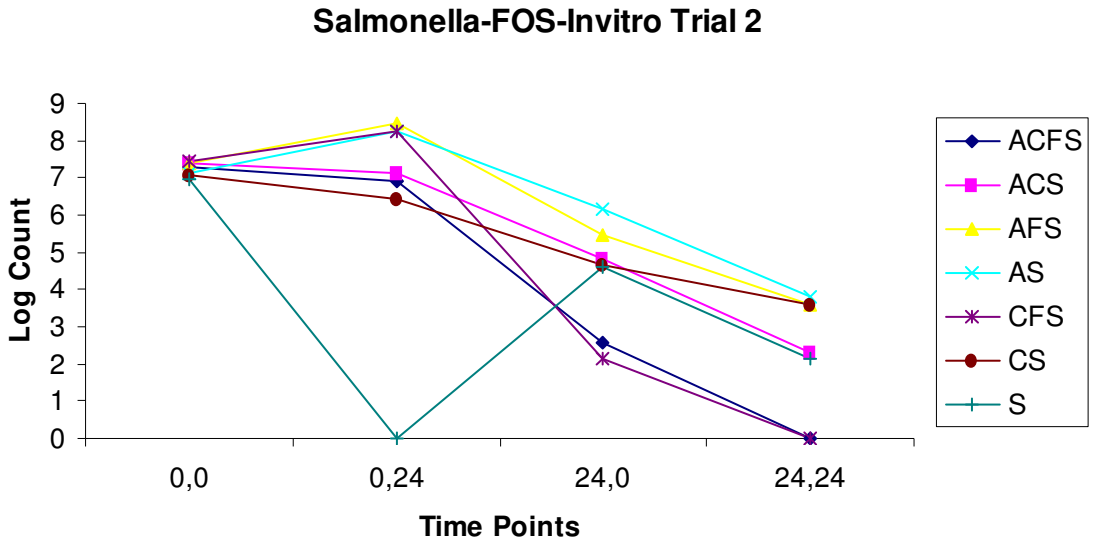

Figure A-4. Effects of *in vitro* fermentation with hen cecal contents on *Salmonella* Typhimurium growth when *Salmonella* was inoculated at 0 hr *in vitro* fermentation (Trial 2).

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